

January 2009

MUTATION SCREENING OF CANDIDATE BREAST CANCER SUSCEPTIBILITY GENES IN NON-BRCA1/2 FAMILIES

Cara C. Schafer

Worcester Polytechnic Institute

Follow this and additional works at: <https://digitalcommons.wpi.edu/mqp-all>

Repository Citation

Schafer, C. C. (2009). *MUTATION SCREENING OF CANDIDATE BREAST CANCER SUSCEPTIBILITY GENES IN NON-BRCA1/2 FAMILIES*. Retrieved from <https://digitalcommons.wpi.edu/mqp-all/162>

This Unrestricted is brought to you for free and open access by the Major Qualifying Projects at Digital WPI. It has been accepted for inclusion in Major Qualifying Projects (All Years) by an authorized administrator of Digital WPI. For more information, please contact digitalwpi@wpi.edu.

**MUTATION SCREENING OF CANDIDATE BREAST CANCER
SUSCEPTIBILITY GENES IN NON-BRCA1/2 FAMILIES**

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

Cara Schafer

January 14, 2009

APPROVED:

Csilla Szabo, Ph.D.
Mayo Graduate School College of Medicine
Dept of Biochemistry and Molecular Biology
Project Advisor

David Adams, Ph.D.
Biology and Biotechnology
WPI Project Advisor

ABSTRACT

Although mutations in BRCA1 and 2 genes have previously been linked to breast cancer, mutations in genes encoding other proteins in the BRCA DNA repair pathway could also lead to this disease. This MQP used a candidate gene screening approach to identify potential genetic changes in proteins previously shown to interact with BRCA1 and/or BRCA2 in repair pathways. PCR amplicons were analyzed by high resolution melting analysis (HRMA) as a preliminary screen for mutations in six candidate genes (Mre11, Rad50, MCPH1, NBS1, DSS1, and BCCIP) amplified from non-BRCA1/BRCA2 breast cancer patient samples from BRCA-independent high-risk families. Mutations in MCPH1 were further analyzed by DNA sequencing, which showed frameshift/nonsense mutations, missense mutations, silent substitutions, and intronic variants in 29 patients, 10 of which contained more than one mutation. Mutations in exon-2 of DSS1 include protein truncating and missense mutations in highly conserved domains.

TABLE OF CONTENTS

Signature Page	1
Abstract	2
Table of Contents	3
Acknowledgements	4
Background	5
Project Purpose	26
Methods	27
Results	34
Discussion	57
Bibliography	59
Appendix.....	66

ACKNOWLEDGEMENTS

I would like to thank the following individuals for their kind assistance and support:

- Dr. Csilla Szabo, my advisor and mentor, for allowing me to work with her. I wish to express my gratitude for her inspiration and guidance, and for her enthusiasm and encouragement throughout the MQP.
- Dr. Fergus Couch for his support of this project.
- Bruce Eckloff and the Mayo DNA Sequencing Facility for providing the sequencing data of the variants.
- Chris Hilker and the Genotyping Core for instruction and use of Pre-PCR robotics.
- Dr. David Adams for his assistance initiating the project, for his constructive comments and suggestions, and for his help editing the final MQP report.

BACKGROUND

Breast Cancer Description

Breast cancer is a malignancy that develops in tissues of the breast, usually in the ducts and lobules. It occurs in a greater percentage in females, and rarely in males (National Cancer Institute, 2007). Some of the first physically identifiable symptoms of breast cancer include a lump or swelling, skin dimpling, nipple pain, discharge, or retraction, redness, or scaliness of the nipple or breast skin (American Association for Cancer Research, 2007).

Mammography is an effective and widely used diagnostic tool to detect changes in breast tissue that may indicate cancer. Other diagnostic methods used are digital mammography, magnetic resonance imaging, positron emission tomography, Sestamibi scintimammography, and ductal lavage (National Cancer Institute, 2007). Family history of breast cancer is the greatest predictor of risk for developing the disease. Other factors include age, previous history of breast cancer, history of chest radiation therapy before age 30, and existence of having dense breast tissue, particularly in older individuals. Women with a history of early onset of menses or late age menopause, women who have never had children or who have children after age 30, women who are obese after menopause, or who use menopausal hormone therapy with estrogen plus progestin are also considered to have increased risk for breast cancer (American Association for Cancer Research, 2007; National Cancer Institute, 2007).

Breast Cancer Prevalence

Breast cancer is the second leading cause of cancer deaths in American women (Friedenson, 2005). In 1997, breast cancer claimed the lives of approximately 44,910 Americans, with 43,900 women and 290 men losing their lives to the disease. Approximately two million women living in the U.S. have been diagnosed with breast cancer. The National Cancer Institute (2007) predicts that in the United States in 2007, there will be 178,480 newly diagnosed cases of breast cancer in females, and 2,030 new cases in males. Over 40,000 people will die from breast cancer in 2007 (40,460 females and 450 males) (National Cancer Institute, 2007). Mortality rates have, however, decreased an estimated two percent per year in the past decade, with greater decreases seen among young women (American Association for Cancer Research, 2007), possibly due to mammography screening, early diagnosis, and improved management (adjuvant tamoxifen therapy) and treatment of women with breast cancer (Hermon and Beral, 1996).

Risk Reduction Options in Familial Breast Cancer

Familial breast cancer is characterized by early onset diagnosis, an increased risk of bilateral breast cancer, an increasing risk with increasing numbers of affected family members, and increased risk for ovarian cancer. Women who have BRCA1 and BRCA2 gene mutations face this increased risk for breast and ovarian cancer. BRCA1 and BRCA2 account for almost 80% of hereditary breast cancer, and 5 to 6% of all breast cancers (Greene, 1997). At least eight candidate breast cancer susceptibility genes have currently been identified (Greene, 1997) (discussed later). In a study conducted by the

Breast Cancer Linkage Consortium, 52% of families with breast cancer demonstrated linkage to BRCA1, while 32% were linked to BRCA2, suggesting that 16% of breast cancer families were linked to other predisposing genes (Ford *et al.*, 1998). BRCA1 was linked to 81% of breast-ovarian cancer families, while 14% were linked to BRCA2. Linkage to BRCA2 was identified in 76% of families with male and female breast cancer (Ford *et al.*, 1998). The cumulative risk of breast cancer was 27% by age 50 years, which increased to 84% by age 70 years. Ovarian cancer risks were much smaller, at 0.4% until age 50 years, but rose to 27% by age 70 years (Ford *et al.*, 1998).

Some risk-reduction options are made available, as part of genetic counseling for women who are BRCA1 or BRCA2 mutation carriers. Options offered include increased surveillance, chemoprevention with tamoxifen, prophylactic oophorectomy (removal of the ovaries), and prophylactic mastectomy (Uyei *et al.*, 2006).

Uyei *et al.* (2006) reported a retrospective analysis of 554 women with BRCA1 and BRCA2 gene mutations who were treated at The University of Texas M. D. Anderson Cancer Center. Results obtained for data collected between 2000 and 2006 demonstrated that women who had BRCA mutations, along with a history of breast cancer or ductal carcinoma *in situ*, or a history of having had previous breast biopsies, were more likely to select prophylactic surgery. Women with a family history of ovarian cancer opted for prophylactic oophorectomy, while an individual's personal history of ovarian cancer or advanced breast cancer was more likely associated with a choice for surveillance only. Breast cancer survivors with a history of treatment with total mastectomy chose prophylactic mastectomy more often than breast cancer survivors with a history of treatment with breast-conserving surgery or women with no history of breast cancer

(Uyei *et al.*, 2006). Hartmann *et al.* (2001) reported that bilateral prophylactic mastectomy decreased the risk of breast cancer in women with BRCA1 and BRCA2 mutations by approximately 90%.

Researchers at Lombardi Comprehensive Cancer Center in Washington, DC reported that BRCA1 and BRCA2 carriers are recommended to undergo prophylactic bilateral salpingo-oophorectomy (removal of an ovary with a fallopian tube) by age 35-40 years or when childbearing is complete, in an effort to significantly reduce the risk of ovarian cancer (Nusbaum and Isaacs, 2007). This prophylactic surgery has been shown to also reduce the risk of breast cancer when performed in premenopausal mutation carriers. Finch *et al.* (2006) studied the incidence of ovarian, fallopian tube, and primary peritoneal cancer in a large cohort of women with BRCA1 or BRCA2 mutations. Prophylactic oophorectomy reduced the risk of ovarian and fallopian tube cancer in the *BRCA1* and *BRCA2* carriers by approximately 80%, although there was a post-oophorectomy residual risk of approximately 4% to develop peritoneal cancer (Finch *et al.* 2006). BRCA1 and BRCA2 carriers are also offered the option of increased surveillance, with or without chemoprevention, or prophylactic surgery as part of a breast cancer management protocol. Bilateral prophylactic oophorectomy is more commonly chosen than bilateral prophylactic mastectomy in BRCA1/2 mutation carriers who are unaffected (Freibel *et al.*, 2007), as many women feel that bilateral prophylactic mastectomy is too aggressive with increase risk for side effects (Uyei *et al.*, 2006).

BRCA carrier status, to date, is not used as an independent factor to determine prognosis for systemic treatment options (Nusbaum and Isaacs, 2007). Recently, researchers have investigated the use PARP-1 [poly(ADP-ribose) polymerase-1]

inhibitors as a chemotherapeutic treatment for BRCA1/2 cancers. DeSoto and Deng (2006) suggested that BRCA breast cancer cells were resistant to PARP-1 inhibitors when used alone in the treatment. PARP-1 inhibitors did offer promise in the prevention of BRCA related breast cancers, and may be successful when used in combination with other chemotherapeutic agents in the treatment of BRCA related breast cancer (DeSoto and Deng, 2006).

Genetic Causes of Breast Cancer

BRCA1 and BRCA2

Approximately 5-10% of all breast cancer results from the inheritance of highly penetrant mutations in two susceptibility genes, BRCA1 (OMIM, 113705; GenBank, U14680.1; Hall *et al.*, 1990) and BRCA2 (OMIM, 600185; GenBank, U43746.1; Wooster *et al.*, 1994) which is consistent with an autosomal dominant transmission (Ford *et al.*, 1998; Pohlreich *et al.*, 2005). These genes were first identified in 1994 (Miki *et al.*, 1994; Wooster *et al.*, 1994; Breast and Ovarian Cancer, 2007), and are associated with both breast and ovarian cancers (Troudi *et al.*, 2007). Mutations in these two genes account for 60% of all known mutation site-specific female breast cancers (Ford *et al.*, 1998). BRCA1 is found on the long arm of chromosome 17, mapped specifically to chromosome 17q21 (Hall *et al.*, 1990). The BRCA1 gene contains 24 exons, and encodes a protein of approximately 220 kDa (1863 amino acids) (Cipollini *et al.*, 2004).

BRCA2 is located on the long arm of chromosome 13. BRCA2 is also a large gene, containing 27 exons that encode a protein of 380 kDa (3418 amino acids). Both BRCA1 and BRCA2 have an unusually large exon 11. The translational start site for both genes is

in exon 2. Both proteins are predominately nuclear, where phosphorylated versions of both proteins are also located (Cipollini *et al.*, 2004). While similar in some respects, many differences exist between the two genes, which are not homologous. BRCA1 has two protein motifs, while BRCA2 has BRC repeats and no relation to BRCA1 (Cipollini *et al.*, 2004) and is not highly conserved evolutionarily (Szabo *et al.*, 1996).

BRCA1 and BRCA2 function as tumor suppressors, and are critical to the cellular control of homologous recombination and double-strand break repair when DNAs are damaged (Liu and West, 2002; Ford *et al.*, 1994; Friedenson, 2005). Individuals with mutations in these genes possess an increased lifetime risk for developing breast or ovarian cancer. The cumulative risk of for developing breast cancer is approximately 28% by age 50 years, and 84% by age 70 years, with ovarian cancer risks determined to be 0.4% by age 50 years and 27% by age 70 years (Ford *et al.*, 1994). The lifetime risk for developing breast cancer is similar in both BRCA1 and BRCA2 carriers, with a possible lower risk in BRCA2 carriers <50 years of age (Ford *et al.*, 1994).

Both BRCA1 and BRCA2, when functioning normally, play an active role in the restoration of double-stranded breaks in DNA caused by radiation, which can occur through exposure to DNA damaging agents(ionizing radiation) or through errors in normal cellular replication (such as DNA synthesis, chromosomal segregation, metabolic generation of oxygen radicals). Inactivating mutations occurring in BRCA1 or BRCA2 hinder the repair of DNA damage through homologous recombination. The accumulation of mutations due to impaired DNA repair promotes the growth of cancer (Breast and ovarian cancer, 2007). Researchers have reported that BRCA1 mutations also confer modest risks for uterine, cervical, early-onset prostate and pancreatic cancers. BRCA2

mutations show a similar increased risk for prostatic, pancreatic, gallbladder, bile duct, stomach cancers and melanoma (Easton et al., 1995; Friedenson, 2005).

Approximately 2000 distinct sequence variants in BRCA1 and BRCA2 have already been recorded (Breast Cancer Mutation Database, 2007). Both BRCA1 and BRCA2 genes have variants that are uniformly distributed along the entire coding regions. Mutations have also been identified in intronic sequences flanking each exon in both genes, some of which lead to altered splicing.

Breast cancer develops in a multistep process and is influenced by two types of genes, oncogenes and suppressor genes (Osborne *et al.*, 2004). Oncogenes refer to genes that, when activated, can contribute to the development of cancer. Oncogenes produce alterations that cause gain-of-function effects (Osborne *et al.*, 2004). Tumor suppressor genes function in slowing down cell growth, DNA repair, and apoptosis (American Cancer Society, 2005). These genes refer to group of genes whose loss of function promotes malignancy. Germline mutations in breast cancer occur in tumor suppressor genes. Tumor suppressor genes can also contain sporadic acquired somatic mutations. The tumor typically contains a mutation in one allele and a deletion of the remaining allele. In 1971, Alfred Knudson proposed this "two-hit" hypothesis (in reference to retinoblastoma) which stated that both gene alleles must be missing to unmask the malignant phenotype. The activation of an oncogene and the mutation of a tumor suppressor gene can produce changes that contribute to the malignancy. The effects of these alterations are complex due to the high number of changes in a typical case of breast cancer and the interactions of the biological pathways involved (Osborne *et al.*, 2004).

Cancer risks increase from missing BRCA1 or BRCA2 protein sequences or non-functional proteins, most likely caused by frameshift, nonsense, and splice site mutations (Cipollini *et al.* 2004). Figure 1 shows how BRCA1 and BRCA2 act as tumor suppressor genes (deduced from an analysis of tumor specimen DNAs) (copied from Cipollini *et al.* 2004). An initial mutation in one allele (white circle in the figure) leads to a diminished capacity for DNA repair. Eventually, the second non-mutated allele is entirely deleted (figure right side).

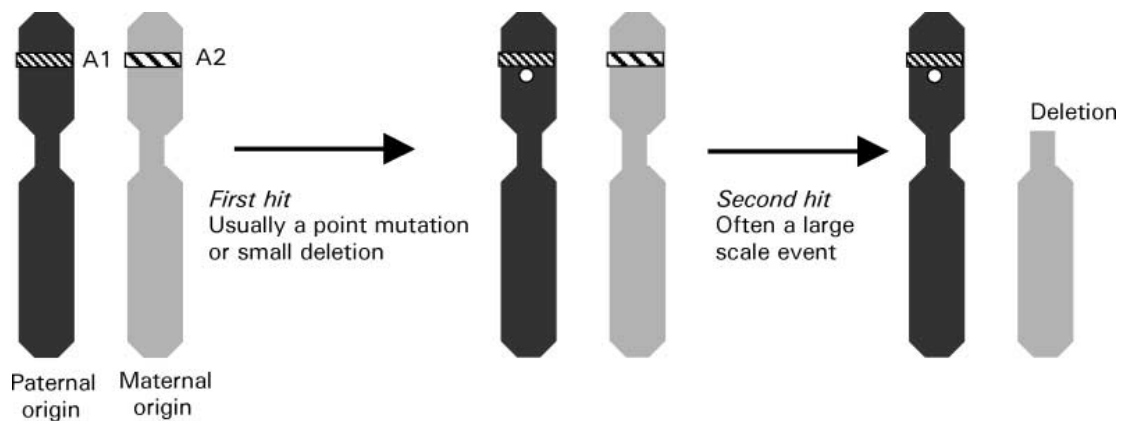


Figure 1. Proposed Mechanism for Loss of Allele Function for BRCA1 and BRCA2 Tumor Suppressor Genes. Initially one allele suffers a germline susceptibility point mutation (white circle), which leads to a diminished capacity for repairing DNA. Eventually the second unmutated allele is deleted entirely, as found in tumor specimens (Copied from Cipollini *et al.* 2004).

The Role of Founder Mutations

The proportional contribution of BRCA1 and BRCA2 mutations has been shown to differ in different populations around the world (Szabo and King, 1997). Vogel *et al.* (2007) described eight different BRCA mutations and three variants within a small sample in a Hispanic population. One of three mutations in the BRCA1 and BRCA2 genes was found in 2.0%-2.5% of Ashkenazi Jewish women (Struwing *et al.*, 1997).

Studies have suggested that each founding mutation put these women at a high risk of invasive breast cancer, which continues throughout life (Struewing *et al.*, 1997; Warner *et al.*, 1999). In addition, an estimated 12% of the total number of breast cancers in the Ashkenazi Jewish population is caused by mutations in the BRCA1 or BRCA2 gene (Warner *et al.*, 1999). Peto *et al.* (1999) reported that the mutation BRCA1-185delAG has been identified in 20% of Ashkenazi Jewish women with early onset breast cancer, while the mutation BRCA2- 6174delT is found in 8% of the Ashkenazi cases diagnosed in women over 42 years of age. The authors speculated that, among Ashkenazi Jews, BRCA1 mutations play a significant role in the risk for early-onset breast cancer, while BRCA2 mutations affect the later onset of the disease. Ganguly *et al.* (1997) found that that lower prevalence of mutations in both BRCA1 and BRCA2 genes was observed from data collected from clinical families. Higher prevalence was found in linkage data obtained from high-risk families collected in a research setting (Ganguly *et al.* 1997).

In Britain, BRCA1 mutation carriers were found in 3.1% of breast cancer patients, and BRCA2 mutations in 3.0% of breast cancer patients under the age of 50 (Peto *et al.*, 1999). For patients 50 years of age or older, the prevalence was 0.49% and 0.84%, respectively. Similarly, researchers in Australia found that an estimated 3.8% of women before age 40 in that country carried a germline mutation in BRCA1 (Southey *et al.*, 1999). They reported seven rare BRCA variants, but argued that these did not have a significant effect on the risk of breast cancer in the population studied.

In a recent study of 204 breast cancer patients in northern India, researchers found a lower proportion of BRCA1 and BRCA2 mutations than seen in other populations, although the proportion was still elevated for breast cancer patients versus the general

population (Saxena *et al.*, 2006). Interestingly, 9 distinct BRCA1 and 9 distinct BRCA2 sequence variants were identified. 4 of the 9 BRCA1 mutations were unique to the Indian population, accounting for 44% of the BRCA1 mutations found. Of the 9 BRCA2 mutations, 7 mutations (78%) were unique to the Indian population, accounting for 78% of the BRCA2 mutations. In this group of patients studied from Northern India, these unique mutations were distributed throughout the BRCA1 and BRCA2 gene exons (Saxena *et al.*, 2006).

Szabo and King (1997) analyzed the results of previous studies conducted by researchers in seventeen countries, including Italy, Finland, Norway, Iceland, Israel, Russia, Japan, Canada, Britain, and the United States. They explained that great variability was noted among populations for proportions of high-risk families with BRCA1 mutations. Russia displayed the largest proportion of BRCA1 mutations, occurring in 79% of families with breast or ovarian cancer. Affected families had one of two common alleles. The most common allele in Russia was BRCA1-5382insC, which is also the most common allele found among the Europeans studied. The second most common allele in Russia was BRCA1-4153delA (Szabo and King, 1997). This allele has also been identified in affected families in Latvia (Csokay *et al.*, 1999), Poland (Gorski *et al.*, 2000; Gorski *et al.*, 2004), and Lithuania (Gronwald *et al.*, 2005). Israel demonstrated the next highest proportion of BRCA1 mutations in inherited breast and ovarian cancer, occurring in 47% of high-risk families (Szabo and King, 1997). BRCA1 mutations were observed in 29% of Italian high-risk families, and in 20-25% of high-risk families in Britain, France, Scandinavia, and Hungary. Less than 20% of high-risk families in Holland, Belgium, Germany, Norway, and Japan had BRCA1 mutations.

Significantly fewer cases of BRCA2 mutations were noted for all countries except Iceland, where a single BRCA2 mutation, specifically the 999del5 mutation, was responsible for all the inherited breast and ovarian cancer in that country.

BRCA2 mutations are also more common than BRCA1 mutations in familial male breast cancer, occurring in about 19% of familial male breast cancer in the United States (Szabo and King, 1999). Overall, the authors proposed that “BRCA1 and BRCA2 have each undergone multiple mutations; the resultant alleles have migrated with the peoples in which they occur; and disease-associated mutations have persisted, no doubt because of late onset of disease and, hence, little or no deleterious impact of mutant alleles on genetic fitness” (Szabo and King, 1997).

Non-BRCA Genes Associated with Breast Cancer

Recent studies suggest that the proportion of familial breast cancer cases due to the *BRCA1* and *BRCA2* mutations may be smaller than initially believed (Kainu, *et al.*, 2000). Mutations in *BRCA1* and *BRCA2* account for only about 60% of mutation site-specific female breast cancers (Ford *et al.*, 1998), so additional susceptibility genes likely exist (Walsh and King, 2007). But to date, gene identification efforts using linkage analysis have not been successful at identifying non-BRCA genes, likely because that approach identifies individual genes, each of which confers only a moderate risk (Antoniou and Easton, 2006).

Kainu *et al.* (2000) used mathematical models to look for early somatic genetic deletions in tumor tissues, and then applied targeted linkage analysis. The authors used comparative genomic hybridization to investigate 61 breast tumors from 37 breast cancer

families, none of whom had *BRCA1* or *BRCA2* mutations. Mathematical models predicted a loss of chromosome arm 13q as one of the first genetic events in these familial cancers. This was demonstrated in a study of a Swedish family with five breast cancer cases, where all patients evidenced clear 13q deletions at 13q21-q22 (Kainu, *et al.*, 2000). A subsequent study by Thompson et al., (2002) found no linkage to a susceptibility locus at chromosome 13q21 and concluded that, if it did exist, its contribution would be minimal in breast cancer families of European origin.

There exist multiple biologic functions for BRCA1 and BRCA2 proteins, including “participating within a pathway that mediates error-free repair of DNA double stranded breaks by homologous recombination (Friedenson, 2005).” BRCA1 and BRCA2 gene products are placed within a biochemical sequence, which includes the MRE11, Rad50 and NBS1 complex (MRN complex), ATM, CHEK2, BRCA1, BRCA2, and Fanconi anemia proteins, often referred to as the BRCA pathway (Figure-2, Friedenson, 2007). A breakdown in the critical protein function anywhere within this DNA repair BRCA pathway may introduce mutations by repair of double strand breaks by lower fidelity, error prone methods. Because some cancers are mediated by these errors, this results in an increased risk for development of those cancers (Friedenson, 2005). More recently, Friedenson (2007) proposed that inactivation of any component within the BRCA pathway would increase the risks for not only breast and ovarian cancers, but also for lymphomas and leukemias. Where BRCA pathway mutations do not exist, the functional encoded proteins provide protection from both breast and ovarian cancer.

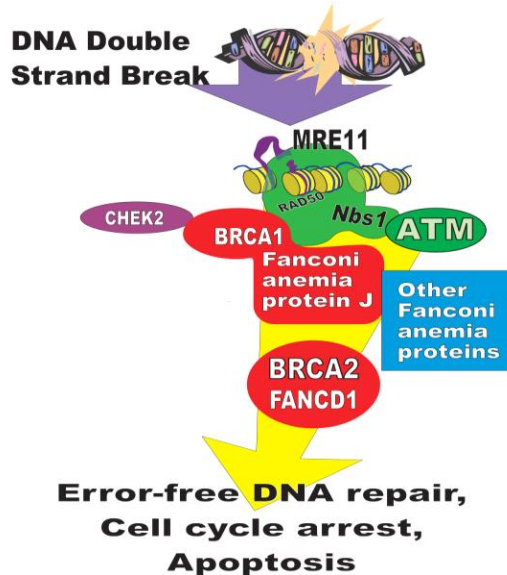


Figure 2. Schematic Diagram of the BRCA Pathway for DNA Repair. Note that BRCA1 and BRCA2 are only a part of this key DNA repair pathway, which if any key protein is rendered non-functional by mutation, DNA mutations subsequently increase. Thus this model predicts non-BRCA mutations should also correlate with breast cancer. (Figure from Friedenson, 2007)

Gene mutations cause inactivation of BRCA1, BRCA2, and other critical proteins within this "BRCA pathway" that inactivate this error-free repair process (Friedenson 2007). Liu and West (2002) described the pathway, which illustrates how, even though BRCA1 and BRCA2 proteins interact together, only a minority of the BRCA1 protein is actually found in association with BRCA2 at a given time. Recent identification of more proteins that associate with either BRCA1 or BRCA2 further emphasizes that BRCA1 and BRCA2 each participate in different protein complexes, and these each have distinct functions in DNA double strand breakage repair (See Figure 3 and Table I below).

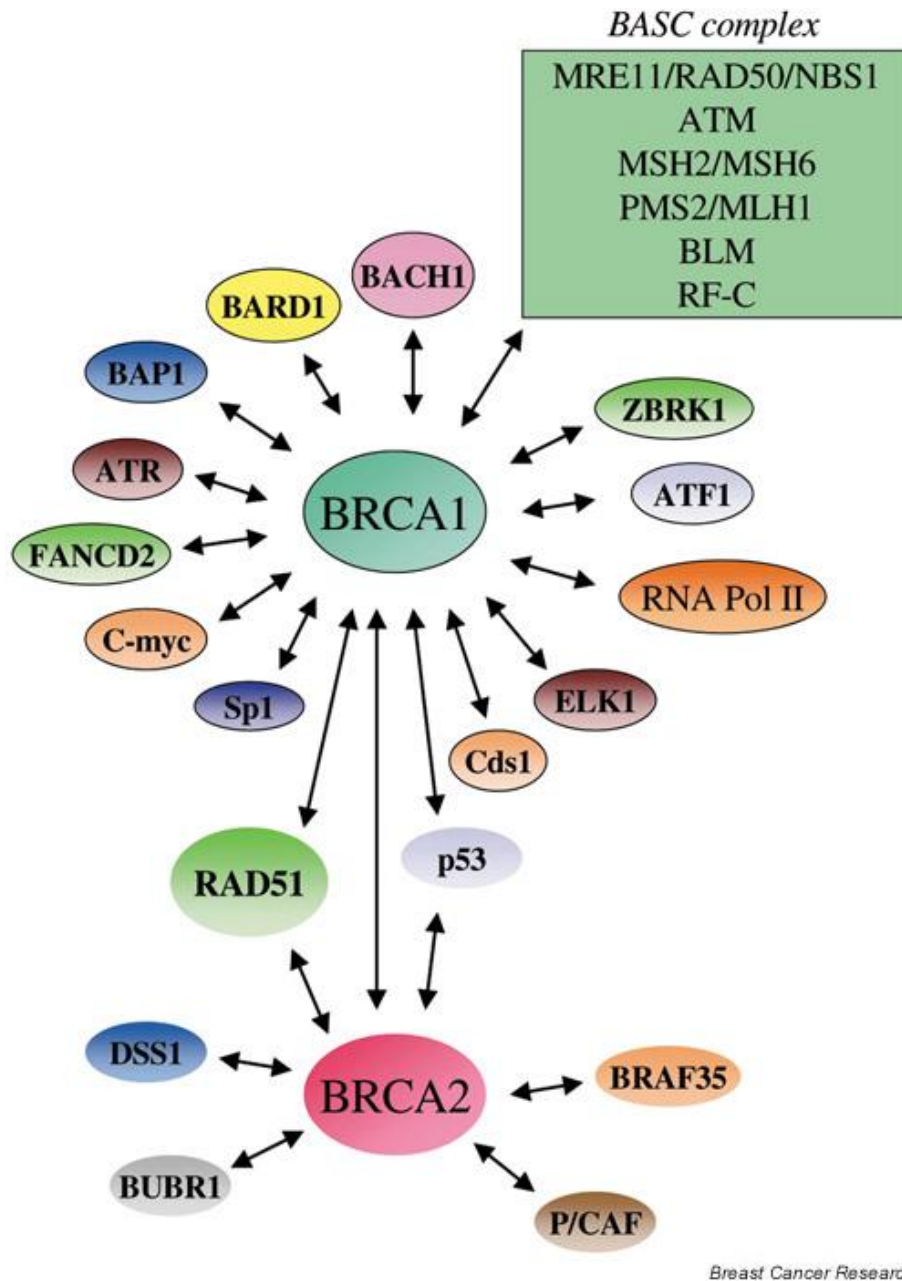


Figure 3. Interactions of BRCA1 and BRCA2 With Other Proteins, or Protein Complexes. These proteins were identified by two-hybrid screens, co-immunoprecipitation analyses, and co-fractionation studies. In general, the protein–protein associations of BRCA1 have been shown to be distinct from those exhibited by BRCA2. (Copied from Liu and West, 2002)

Biological Functions	BRCA-1-Interacting Proteins
DDR and repair	MSH2, MSH6, MLH1, ATM, BLM and the RAD50-MRE11-NBS1, DNA replication factor C, RAD51, Fanconi anemia proteins, PCNA, H2AX, c-Abl, MDC1
Tumor suppressors	ATM, ATR, p53, BRCA2, RB, BARD1, BACH1
Oncogenes	c-Myc, casein kinase II, E2F1, E2F4, STK15, AKT
Transcription	RNA polymerase II holoenzyme (RNA helicase A, RPB2, RPB10 α), CBP/p300, HDC and CtIP, estrogen receptor α , androgen receptor, ZBRK1, ATF1, STAT1, Smad3, BRCT-repeat inhibitor of hTERT expression (BRIT1)
Cell cycle related	Ayclin A, Cyclin D1, Cyclin D1, CDC2, Cdk2, Ckd4, γ -tubulin, p21, p27
Stress response, apoptosis	MEKK3, IFI16, X-linked inhibitor of apoptosis protein (XIAP)
Others	BAP1, BIP1, BRAP2, importin α

Table 1. A list of BRCA1 Interacting Proteins. (Copied from Deng, 2006).

CHEK2 (OMIM 604373) (shown as purple in Figure-2, but not shown in Figure-3 or in Table-I) is a key checkpoint kinase of the BRCA pathway that serves as a tumor suppressor in response to DNA double-strand breakage. The CHEK2*1100delC mutation most likely accounts for familial risk of breast cancer in some non-BRCA1 and non-BRCA2 patients, causing DNA damage and activation of cell-cycle checkpoints that block cell proliferation and DNA repair. The impaired function of these checkpoints results in instability in the genome and a subsequent increased risk for cancer (Weischer *et al.*, 2007). Investigators in Denmark reported the results of a 34 yearlong study of a large sample in the Danish population (Weischer *et al.*, 2007). The authors concluded that CHEK2*1100delC heterozygosity was associated with a three-fold risk of breast cancer in the Danish women studied. Conversely, researchers investigating a small sample of hereditary breast and ovarian cancer families from the Slovak Republic did not

detect any 1100delC variant of the CHEK2 gene (Cierniková *et al.*, 2005). Instead, the investigators found a spectrum of eight mutations, one novel BRCA1 deletion, and one recurrent BRCA2 mutation.

Other candidate genes have been found to act directly in double strand DNA break repair. Walsh, *et al.* (2006) studied 300 US families with 4 or more cases of breast or ovarian cancer who tested negative (wild type) for BRCA1 and BRCA2 mutations. Patients were screened for genomic rearrangements in BRCA1 and BRCA2, and germline mutations in CHEK2, TP53, and PTEN. Based on their findings, these researchers estimated that, in a similar cancer population, one might expect that approximately 12% would demonstrate a large genomic deletion or duplication in either BRCA1 or BRCA2, and that 5% would carry a mutation in CHEK2 or TP53. Recently, several mutations in genes in the BRCA-related pathways (Chek2, ATM, PALB2, BRIP1) were shown to be associated with familial breast cancer (Walsh and King, 2007).

Besides BRCA1 and BRCA 2, other genetic syndromes are associated with autosomal dominant inheritance of breast cancer risk. These include Li–Fraumeni syndrome (a genetic disorder that causes breast cancer), as well as bone cancer (osteosarcoma), muscle and soft tissue cancers, brain tumors, leukemias, and cancer of the adrenal glands. These are caused by germline mutations in another key tumor suppressor gene, p53, found in over 50% of families, with a reported penetrance of at least 50% by age 50 years. Germline mutations in hCHK2 and TP53 genes have also been associated with the Li-Fraumeni syndrome and related breast cancer (Cipollini *et al.*, 2004; Li-Fraumeni Syndrome, 2007). Cowden syndrome is caused by PTEN germline mutations that promote an increased risk for not only developing breast cancer, but also

thyroid and endometrial cancer (lining of the uterus). In addition, patients with Cowden syndrome are at risk for developing noncancerous breast and thyroid diseases, as well as growths on the skin and mucous membranes called hamartomas (Cipollini et al., 2004; Cowden Syndrome, 2007).

Approximately 1% of the general population may be heterozygote carriers of an ATM gene mutation responsible for ataxia telangiectasia, a genetic autosomal recessive disorder, with known risks for developing breast cancer (Cipollini et al., 2004). Another identified autosomal recessive disorder, Peutz–Jeghers syndrome is characterized by early onset of symptoms, which include hamartomatous polyps in the gastrointestinal tract (Peutz–Jeghers Syndrome, 2007). Patients with Peutz–Jeghers syndrome face lifetime risks for cancers of the gastrointestinal tract, pancreas, cervix, breast, and ovaries (Cipollini *et al.*, 2004; Peutz–Jeghers syndrome, 2007). Approximately half of the patients with Peutz–Jeghers syndrome have mutations in STK11, which place them at very high risk of developing breast cancer (Cipollini et al., 2004).

A recent study in Montreal found a high penetrance of PALB2 mutations in probands (initial subjects tested) tested in 68 BRCA1/BRCA2-negative breast cancer families of Ashkenazi Jewish, French Canadian, or mixed ethnic descent (Tischkowitz *et al.*, 2007). Seal *et al.* (2006) looked at truncating mutations in the Fanconi anemia J gene BRIP1 in BRCA1/BRCA2 mutation-negative families. The authors reported that these BRIP1 mutations could pose a risk as low-penetrance breast cancer susceptibility alleles. However, other investigators were unable to substantiate an increase in risk of familial breast cancer from BRIP1 variants (Lewis et al., 2005; Frank *et al.*, 2007). Researchers in Finland analyzed the Mre11 complex, composed of RAD50, NBS1 and MRE11 and

found that RAD50 and NBS1 haplo-insufficiency affected genomic integrity and increased susceptibility to breast cancer (Heikkinen *et al.*, 2006). The MRN complex is comprised of MRE11, RAD50, and NBS1, which is the product of Nijmegen breakage syndrome (Robert *et al.*, 2006). Persons with Nijmegen breakage syndrome (NBS) are susceptible to immunodeficiency and increased risk of malignancies (Tauchi *et al.*, 2002). The NBS1 gene product, nibrin, along with the rest of the MRN complex, is responsible for detecting, signaling and repairing double strand breaks in DNA, and acts as a sensor to recruit ATM to repair broken DNA molecules (Robert *et al.*, 2006).

Li *et al.* (2006) recently investigated the protein DSS1 and described it as an evolutionarily conserved acidic protein that binds to BRCA2. The authors explained that DSS1 depletion causes hypersensitivity to DNA damage, similar to that seen with BRCA2. They found that the presence of DSS1 was essential to the stability of the BRCA2 protein in mammalian cells. Deletion, suppression, or mutation of DSS1 is speculated to promote human breast and ovarian cancer, as well as sporadic and familial breast cancer where BRCA1 and BRCA2 mutations are not present (Li *et al.* 2006). Lu *et al.* (2007) recently reported the results of their study, which showed that BCCIP regulates homologous recombination and suppresses spontaneous DNA damage. They proposed that BCCIP fragments that interact with BRCA2, or with the protein interacting p21, inhibit DNA double-stranded break repair through homologous recombination.

When breaks in DNA structure occur, responses at the cellular level set off numerous checkpoint and repair proteins. These responses synchronize a complex signaling cascade that detects the DNA damage, with subsequent checkpoint activation, DNA repair, cell cycle arrest and/or apoptosis (Chaplet *et al.* 2006). Lin *et al.* (2005)

studied the function of BRIT1 in DNA damage checkpoints. BRIT1 is identical to the MCPH1 gene. Mutations in this gene are also found in patients with primary microcephaly. BRIT1 is required for the expression of both BRCA1 and the checkpoint kinase. Chk1 and phosphorylation of Nbs1 are dependant on BRIT1/MCPH1. The authors speculated that since BRIT1/MCPH1 regulates Nbs1, BRCA1, and Chk1, defects in BRIT1 will likely cause checkpoint defects. Rai *et al.* (2006) proposed that BRIT1 levels contribute to tumor progression by increasing the instability of the gene and promoting metastasis. The authors emphasized that previous studies had shown that BRIT1 expression was inversely correlated with the likelihood of breast cancer metastasis and with the duration of relapse-free survival. The authors found decreases in BRIT1 in the breast and ovarian cancer specimens studied.

The action of BRIT1 in signaling DNA damage is organized in a hierarchical fashion as seen in Figure 4. The model proposed by Chaplet *et al.* (2006) suggests that BRIT1 involvement begins early in the recruitment, and subsequent triggering of DNA damage induces early mediators in the repair pathway.

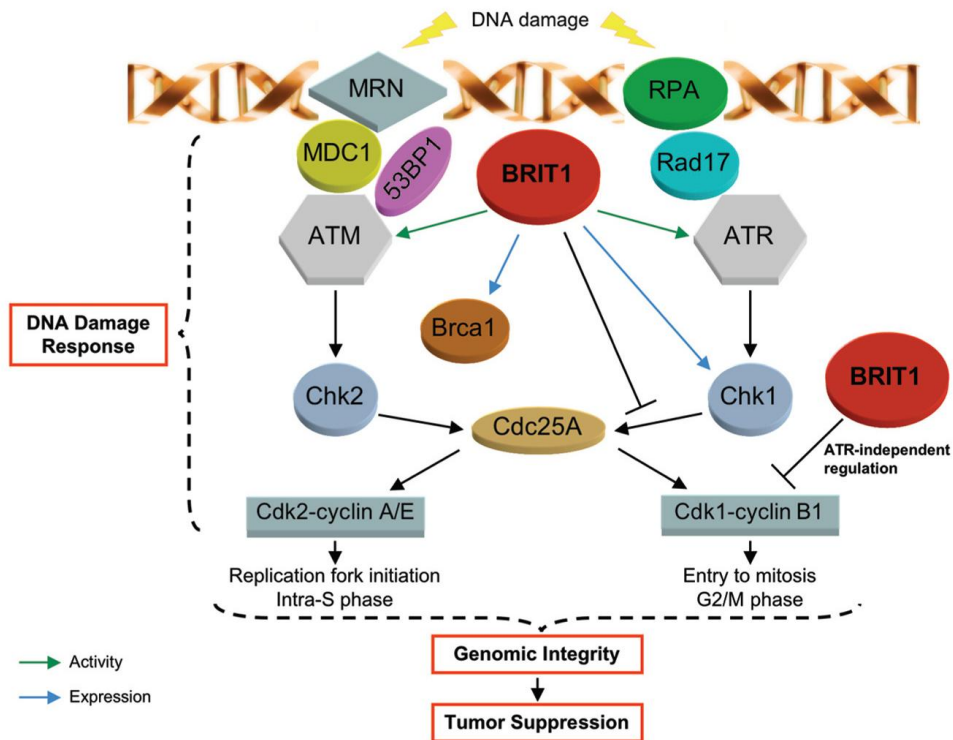


Figure 4. A Model of BRIT1 Functions in DNA Damage Response, Genomic Integrity, and Tumor Suppression. BRIT1 is an early regulator of the DNA damage response in both ATM and ATR pathways, and is required for the expression of BRCA1 and Chk1. BRIT1 also functions as an adaptor downstream of Chk1 in the ATR pathway, and is essential for maintenance of genomic integrity and tumor suppression.” (Copied from Chaplet *et al.*, 2006)

Chaplet *et al.* (2006) contend that BRIT1 may also be necessary to preserve an intact chromatin structure, which is also essential to the DNA damage checkpoint and repair machinery. BRIT1 depletion eliminates the DNA damage checkpoint and repair response, causing both centrosomal defects as well as chromosomal aberrations. Human carcinomas have been shown to have aberrantly reduced expression of BRIT, suggesting that BRIT1 plays a role in the development and progression of cancer, further supporting the role of BRIT1 as a tumor suppressor (Chaplet *et al.*, 2006).

Although biochemical analyses have shown that multiple proteins participate in the BRCA pathway for the repair of double stranded DNA breaks, and a loss of their

function should hypothetically correlate with an increased risk of breast or ovarian cancer, to date, gene identification efforts using linkage analysis have not been successful at identifying non-BRCA genes. This is likely because that approach identifies individual genes, each of which confers only a moderate risk (Antoniou and Easton, 2006). Perhaps a direct candidate gene sequencing effort which targets known BRCA pathway-associated genes will succeed.

PROJECT PURPOSE

The overall objective of this project was to identify mutations within novel BRCA pathway-associated genes that might contribute to the onset of breast cancer. The lab's main hypothesis is that the genetic loss of any gene related to DNA repair, and in particular those genes known to participate in the BRCA-associated DNA repair pathway, can lead to a loss of DNA repair, and the formation of cancer. This MQP study used a candidate gene screening approach to identify novel susceptibility genes by directly sequencing the coding regions of genes previously shown to interact with BRCA1 and/or BRCA2 in the BRCA DNA repair pathway (Liu and West, 2002). PCR amplicons for genes Mre11, Rad50, NBS1, DSS1, BCCIP, and MCPH1 were amplified from patient DNAs from high-risk non-BRCA1/BRCA2 families. The amplicons were then analyzed by High Resolution Melting Analysis (HRMA) to identify potential variants. Variants for MCPH1 were further analyzed by DNA sequencing.

METHODS

Tissue Source

Germline DNA samples from blood were obtained from 288 subjects with a history of breast cancer. All subjects were patients from high-risk non-BRCA1/BRCA2 families, obtained from various collaborating centers in Europe and the United States.

DNA For PCR Optimization

Human placental DNA (Clontech) was used for PCR optimization.

PCR

Mutation analysis was performed on specific candidate genes known to participate in the BRCA pathway for DNA repair using traditional PCR (not real time PCR) followed by DNA sequencing.

PCR Primer Pair Optimizations

PCR primer pairs were designed using bioinformatics tools for specific gene exons for the following genes: MCPH1, NBS1, DSS1, and BCCIP. Bioinformatics tools included online applications such as Primer3 Input v. 0.4.0., UCSC's ePCR and BLAT, and NCBI's GenBank. NCBI's GenBank was accessed in order to obtain cDNA information for the novel susceptibility genes.

Primer-3 was used to pick the primer pairs, which were then analyzed to confirm that each forward primer melting temperature was similar to the reverse primer melting

temperature, resulting in a more stringent annealing temperature range for the PCR product. By analyzing the Primer3 output, primer conditions could be created to avoid primer dimer formation and check for stem-loop structures. The primer pairs were also designed to include a length of approximately 50 nucleotides on either end of the exon to ensure that the entire coding region would be included. These conditions were necessary for designing primer pairs for mutation screening of all coding sequences and intron and exon boundaries. PCR product size was limited to less than 500 bp for optimal HRMA sensitivity. For large exons, overlapping PCR products were amplified to fit these conditions. UCSC's ePCR and BLAT applications determined each primer pair derived from unique sequence within the human genome to produce a single PCR product.

The new primers were obtained from Integrated DNA Technologies. Optimization of PCR conditions was performed using human placental DNA provided by Clontech for each primer pair, and also for existing primer pairs for MRE11 and RAD50. Optimization was defined as the specific conditions producing the maximum amplicon band intensity without non-specific band amplification. Ideal PCR conditions were determined using a gradient cycler sampling 10 T_m (annealing temperatures) per primer pair. Each reaction contained 20 ng of human placental DNA, PCR master mix (1X Buffer, 1mM MgCl₂, 200 μM dNTPs, 0.5 U Qiagen Hotstar Taq Polymerase), and individual primer pairs (250 uM each) in 96-well Bio-Rad polypropylene Multiplate format. The PCR machine used was a Peltier Thermal Cycler DNAEngine Tetrad2 created by MJ Research. PCR amplification used the general protocol, shown in Table-II, and any change from the general protocol is given in the figure legends.

Table-II: General PCR Conditions Used for Gene Amplification

<i>Step</i>	<i>Temperature</i>	<i>Time</i>	<i>Purpose</i>
<i>1</i>	95°C	15 Min	Initial denaturation and activation of enzyme
<i>2</i>	95°C	30 Sec	Denaturation
<i>3</i>	50-72°C	30 Sec	Temperature gradient annealing
<i>4</i>	72°C	30 Sec	Amplification
<i>5</i>		2 Hrs	Repeat steps 2-4, 39 more times
<i>6</i>	72°C	5 Min	Elongation
<i>7</i>	25°C	∞	Incubation

PCR of Candidate Genes

Optimized PCR conditions for each gene were then implemented on germline DNA from patient blood samples. PCR was performed on white 384-well Bio-Rad Microseal Polypropylene Microplates, each reaction containing 10 ng of non-BRCA1/2 high risk patient DNA. High-throughput liquid handling robotics were used for sample transfer (BIOMEK). White PCR plates were necessary for fluorescence analysis to prevent fluorescence bleed from well to well. The brand of PCR machines used for PCR of the candidate genes included Hybaid Satellite 384 Thermal Cyclers and Thermo Hybaid Multiblock System Software which was programmed for optimal annealing temperatures. The resulting amplicons were then analyzed by using High Resolution Melting Analysis (HRMA) on the Light Scanner (see below).

DNA Amplicon Melting Curve Analysis

As a pre-screen to determine potential sequence mutations, analysis of melting curves from individual amplicons was performed using IdahoTech Light Scanner Hi-Res Melting technology and software. Visibly altered melting curves for patient samples versus non-patient DNA indicated possible sequence variants or heteroduplexes that have different melting temperatures than the normal (wild type or WT) melting temperatures. A heteroduplex is made up of a mismatched, unstable pair of nucleotides (Figure-5). If a dye is used to stain the DNA, its fluorescence decreases for heteroduplexes relative to homoduplexes since less is intercalated.

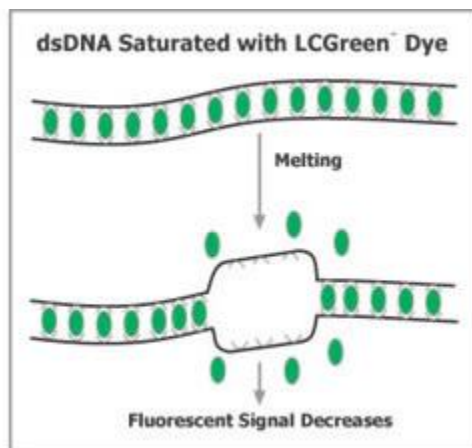


Figure 5. dsDNA Saturated with LCGreen Dye. LG Green Dye enables fluorescence of dsDNA to be monitored during high resolution melting analysis of PCR products (Copied from Idaho Technology Inc., 2007)

Both homoduplexes and heteroduplexes are amplified by PCR during the reaction (Figure-6). The melting temperature of a heteroduplex's dsDNA is lower than the melting temperature of a homoduplex, due to the base mismatches. LC green dye was used to saturate dsDNA, and its fluorescence decreases for a heteroduplex. This technique enabled the fluorescence of dsDNA to be monitored over a temperature range of 45° C to 98° C (Figure-7), and this high resolution melting analysis of PCR products

depicted the various melting curves and peaks which were individually analyzed for possible variants.

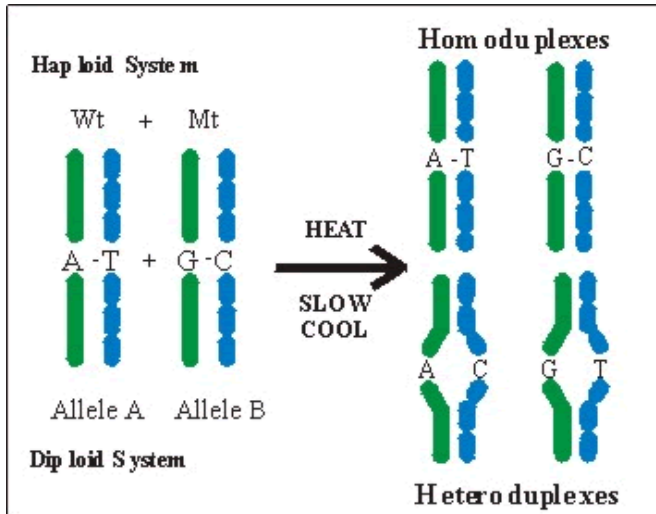


Figure 6. Theory For DNA Melting Analysis. During the reannealing step of PCR amplification, heteroduplexes and homoduplexes of both wildtype and mutant DNA become amplified. (Copied from Cellular and Molecular Biology - DNA Analysis, ncvs.org 2005)

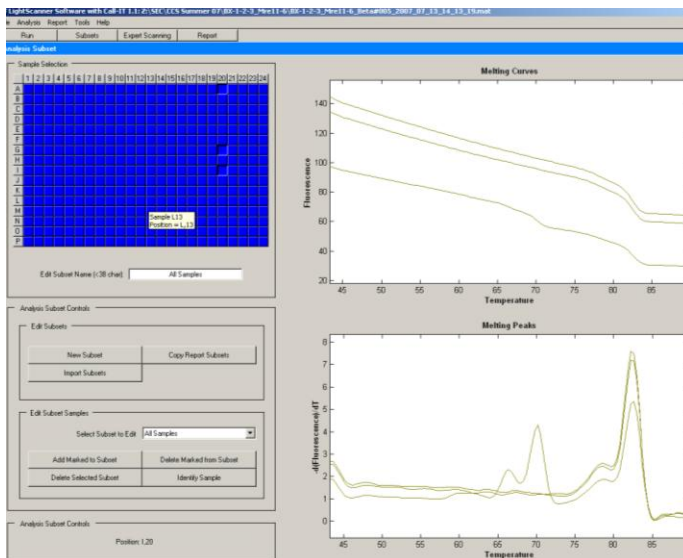


Figure 7. Example of the LightScanner Software Used for the Identification of Mutations and Polymorphisms. Software detects the decrease in fluorescence associated with heteroduplex formation between mismatched nucleotides through fluorescence technology. (Mayo Clinic, 2007, Example of BX-1-2-3_Mre11-6)

DNA Sequence Analysis

PCR amplicons identified from the melting curve analyses as potentially containing mutations were subsequently verified by DNA sequencing. The amplicons of potential variants were collected and transferred to a 96-well plate, on which the ExoSAP-IT protocol was performed to remove excess nucleotides and primers prior to robotic sequencing. 5 μ L of molecular grade water and 2 μ L of ExoSAP-IT were added to each reaction amplicon. The samples were then placed on the PCR machine for PCR clean up. The steps in the ExoSAP-IT protocol are as follows:

1. Incubate at 37° C for 20 minutes.
2. Incubate at 85° C for 20 minutes.
3. Incubate at 25° C forever.

5 μ L of the reaction from the original plate was then transferred to a new 96 well plate. Either the forward or reverse primer was then added to the corresponding amplicons and then placed on the PCR machine for a 5 minute 95° C denature period. The plate was removed and the samples were transferred to a clear 96 well plate required for sequencing. The plate was sent to the Mayo Sequencing Core to be processed.

The ExoSAP-IT reagent, obtained from the USB Corporation, provided a one step PCR clean up that removes excess primers and dNTPs, in preparation for robotic sequencing. The plates were then sent to the core sequencing center at the Mayo Clinic where sequence results were obtained. These results were analyzed for specific mutations, including insertions, deletions, or frameshifts. The samples with mutations were traced back to the initial DNA sample, so that the mutation could be identified along with the individual's family pedigree. Sequencing software used to detect these genetic

mutations included Applied Biosystems' Sequence Scanner v1.0 and Gene Codes' Sequencher 4.8. NCBI's dbSNP was accessed to compare mutations found within the novel susceptibility genes with those defined in the database as part of the HapMap project.

Data Analysis

In order to determine if the identified mutations were disease specific mutations, a comprehensive analysis was conducted on the conserved domains among various species. These conserved domains have remained unchanged throughout evolution which suggests a vital role in gene functionality. The genetic sequences obtained from the NCBI (National Center for Biotechnology Information) database along with the online bioinformatics tool ClustalW2 were used to align the different species' MCPH1 protein sequences. A percentage of cross species conservation was calculated for each identified mutation.

Data Collection

A detailed list of the genetic mutations identified within the MCPH1 gene was recorded to illustrate the family history, cross species conservation, type of mutation, amino acid change, nucleotide change, location of the mutation, minor allele frequency (MAF), observation frequency, and genotype of each sample analyzed.

RESULTS

The purpose of this project was to identify mutations in novel BRCA pathway-associated genes that could contribute to the onset of breast cancer. The assumption was that the genetic loss of any gene related to this important DNA repair pathway could potentially lead to cancer, so breast cancer patients not showing the traditional BRCA1 or 2 mutations might show mutations in BRCA-related genes. Six BRCA-pathway genes were identified as candidates: Mre11, Rad50, NBS1, DSS1, BCCIP, and MCPH1. Mutations in these candidate genes were initially identified using High Resolution Melting Analysis (HRMA) (LightScanner analysis) of PCR amplicons of individual exons amplified from the DNA of non-BRCA1/BRCA2 breast cancer patient samples. Positives for MCPH1 were further analyzed by DNA sequencing.

Primer Optimization on Human Placental DNA

The project was initiated by designing PCR primers for each exon of the six candidate genes, then optimizing the PCR conditions for each primer pair using commercially obtained human DNA. The optimum conditions determined for each primer pair are shown in Table-III below. Figures 8-13 show example PCR gels for the optimization of the primers for MCPH1. Tables IV and V show a typical 96-well format for the PCR reactions with a primer annealing temperature gradient established across the plate.

Table-III: Optimized PCR Conditions for Each Candidate Gene Tested.

<i>Gene Candidate</i>	<i>PCR Condition Changed From the General Protocol in Table-I</i>
<i>MCPH1</i>	
<i>Exon-1</i>	Strongest amplification at 66°C
<i>Exon-2</i>	Strongest amplification between 51-63°C (55°C)
<i>Exon-3</i>	Strongest amplification between 52-63°C (56°C)
<i>Exon-4</i>	Strongest amplification between 56-63°C (59°C)
<i>Exon-5</i>	Strongest amplification between 56-66°C (61°C)
<i>Exon-6</i>	Strongest amplification between 54-66°C (59°C)
<i>Exon-7</i>	Strongest amplification between 54-66°C (59°C)
<i>Exon-8a</i>	Strongest amplification at 63°C (63°C)
<i>Exon-8b</i>	Strongest amplification between 52-66°C (58°C)
<i>Exon-8c</i>	Strongest amplification between 52-66°C (56°C)
<i>Exon-8d</i>	Strongest amplification between 59-69°C (64°C)
<i>Exon-8e</i>	Strongest amplification between 56-63°C (59°C)
<i>Exon-8f</i>	Strongest amplification between 54-66°C (59°C)
<i>Exon-8g</i>	Strongest amplification between 56-66°C (61°C)
<i>Exon-8h</i>	Strongest amplification between 60-63°C (63°C)
<i>Exon-9</i>	Strongest amplification between 54-66°C (63°C)
<i>Exon-10</i>	Strongest amplification between 51-63°C (55°C)
<i>Exon-11</i>	Strongest amplification between 56-63°C (63°C)
<i>Exon-12</i>	Strongest amplification between 56-63°C (59°C)
<i>Exon-13</i>	Strongest amplification between 63-69°C (66°C)
<i>Exon-14a</i>	Strongest amplification between 59-66°C (63°C)
<i>Exon-14b</i>	Strongest amplification between 56-66°C (61°C)
<i>DSSI</i>	
<i>Exon-1</i>	Strongest amplification between 63-69°C (65 °C)
<i>Exon-2</i>	Strongest amplification at 63°C (63 °C)
<i>Exon-3</i>	Strongest amplification between 59-66°C (65 °C)
<i>NBS1</i>	
<i>Exon-1</i>	In progress.
<i>Exon-2</i>	In progress.
<i>Exon-3</i>	In progress.
<i>MRE11</i>	
<i>Exon-1</i>	In progress.

<i>Exon-2</i>	Strongest amplification between 59-66°C (63°C)
<i>Exon-3</i>	Strongest amplification between 54-59°C (56°C)
<i>Exon-4</i>	Strongest amplification between 54-63°C (58°C)
<i>Exon-5</i>	Strongest amplification between 54-63°C (58°C)
<i>Exon-6</i>	Strongest amplification between 56-66°C (61°C)
<i>Exon-7</i>	Strongest amplification between 50-52°C (51°C)
<i>Exon-8</i>	Strongest amplification between 54-63°C (58°C)
<i>Exon-9</i>	Failed, redesign primers
<i>Exon-10</i>	Strongest amplification between 51-56°C (53°C)
<i>Exon-11</i>	Strongest amplification between 54-63°C (58°C)
<i>Exon-12</i>	Strongest amplification between 56-63°C (59°C)
<i>Exon-13</i>	Strongest amplification between 59-66°C (63°C)
<i>Exon-14</i>	Strongest amplification between 51-63°C (57°C)
<i>Exon-15</i>	Strongest amplification between 56-59°C (58°C)
<i>Exon-16</i>	Strongest amplification between 52-63°C (56°C)
<i>Exon-17</i>	Strongest amplification between 54-59°C (56°C)
<i>Exon-18</i>	Strongest amplification between 52-59°C (56°C)
<i>Exon-19</i>	Strongest amplification between 52-59°C (56°C)
<i>Exon-20</i>	Strongest amplification between 54-63°C (59°C)
<i>RAD50</i>	(Using working stock primers previously designed)
<i>Exon-1</i>	Strongest amplification between 63-69°C (66°C)
<i>Exon-2</i>	Failed, redesign primers
<i>Exon-3</i>	Strongest amplification between 52-56°C (54°C at 50 cycles)
<i>Exon-4</i>	Strongest amplification between 52-56°C (54°C)
<i>Exon-5</i>	Failed, redesign primers
<i>Exon-6&7</i>	Failed, redesign primers
<i>Exon-8</i>	Failed, redesign primers; rerun - strongest 56-59°C
<i>Exon-9</i>	Failed, redesign primers; rerun - redesign
<i>Exon-10</i>	Strongest amplification between 54-59°C (56°C)
<i>Exon-11</i>	Strongest amplification between 51-59°C (54°C)
<i>Exon-12</i>	Failed, redesign primers; rerun – strongest 54°C
<i>Exon-13</i>	Failed, redesign primers; rerun – strongest 54-56°C
<i>Exon-14</i>	Failed, redesign primers; rerun – strongest 52-56°C
<i>Exon-15</i>	Strongest amplification 52-56°C; rerun- strongest 51-54°C (52°C)
<i>Exon-16</i>	Strongest amplification between 51-56°C (54°C)
<i>Exon-17</i>	Failed, redesign primers; rerun – strongest 63°C
<i>Exon-18&19</i>	Failed, redesign primers; rerun – strongest 56-63°C
<i>Exon-20</i>	Failed, redesign primers; rerun – strongest 54-59°C
<i>Exon-21</i>	Strongest amplification 56-59°C (58°C with 3 µL BX-1 DNA)
<i>Exon-22</i>	Strongest amplification 56-59°C (58°C with 3 µL BX-1 DNA)
<i>Exon-23</i>	Strongest amplification between 52-66°C (56°C)
<i>Exon-24</i>	Strongest amplification between 52-59°C (56°C)
<i>Exon-25</i>	Failed, redesign primers; rerun- 59-63°C (weak); rerun - 56°C (strong) (60°C)

BCCIP

<i>Exon-1</i>	Strongest amplification at 59°C
<i>Exon-2</i>	Strongest amplification between 51-59°C
<i>Exon-3</i>	Strongest amplification between 54-59°C
<i>Exon-4</i>	Strongest amplification between 56-63°C
<i>Exon-5</i>	Strongest amplification between 63-66°C
<i>Exon-6</i>	Strongest amplification between 59-66°C

Table-IV: Diagram of the 96-well Coordinates and Annealing Temperature Gradient Used for Primer Optimization Tests.

Temperature Gradient (°C)		50	50.6	51.9	53.5	56	59.2	62.9	66	68.5	70.2	71.5	72
		1	2	3	4	5	6	7	8	9	10	11	12
		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	N/A	N/A
B		B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	N/A	N/A
C		C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	N/A	N/A
D		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	N/A	N/A
E		E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	N/A	N/A
F		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	N/A	N/A
G		G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	N/A	N/A
H		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	N/A	N/A

This table shows the 96-well format used for primer optimization. A temperature gradient ranging from 50-72°C (blue color) was distributed across the 96-well plate (50-70°C for 10 samples of each primer pair).

Table-V: Diagram of the Electrophoresis Lane Loadings.

A/B		C/D		E/F		G/H	
marker		marker		marker		marker	
blank		blank		blank		blank	
blank		blank		blank		blank	
blank		blank		blank		blank	
blank		blank		blank		blank	
B10		D10		F10		H10	
A10		C10		E10		G10	
B9		D9		F9		H9	
A9		C9		E9		G9	
B8		D8		F8		H8	
A8		C8		E8		G8	
B7		D7		F7		H7	
A7		C7		E7		G7	
B6		D6		F6		H6	
A6		C6		E6		G6	
B5		D5		F5		H5	
A5		C5		E5		G5	
B4		D4		F4		H4	
A4		C4		E4		G4	
B3		D3		F3		H3	
A3		C3		E3		G3	
B2		D2		F2		H2	
A2		C2		E2		G2	
B1		D1		F1		H1	
A1		C1		E1		G1	
marker		marker		marker		marker	

The DNA marker and the PCR products were loaded into an agarose gel in the order listed in this table, and electrophoresis was performed. The most optimum primer annealing temperatures were determined by analyzing the intensity of the amplicon bands present in the gel. The marker was loaded into the bottom-most and top-most wells. Row A from the 96-well plate was loaded into every other well on the gel. Row B was then loaded next to the A wells. The same process was followed for rows C-H.

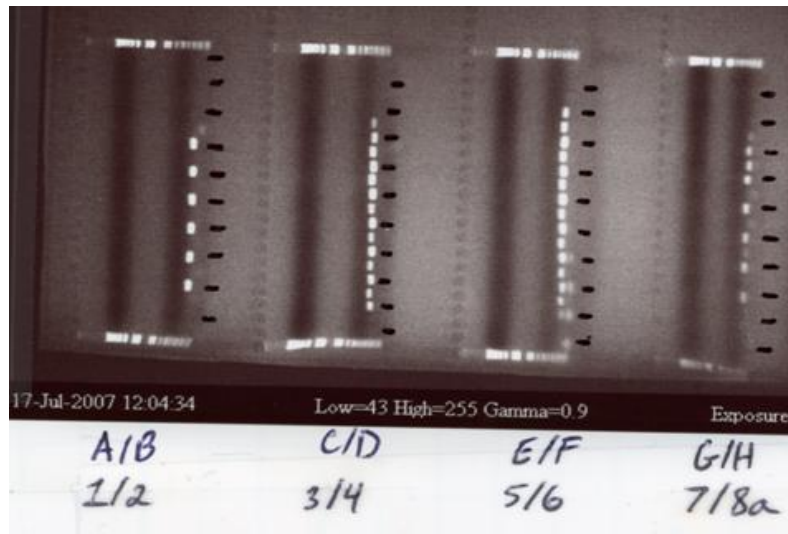


Figure 8. Example of PCR Primer Optimization for MCPH1 Using Human Placental DNA. Figure shows a variety of PCR signals. Primers 1 and 2 (first column), 3 and 4 (second column), and 5 and 6 (third column) show strong amplification, while primers 7 and 8a (right side) show weak amplification.

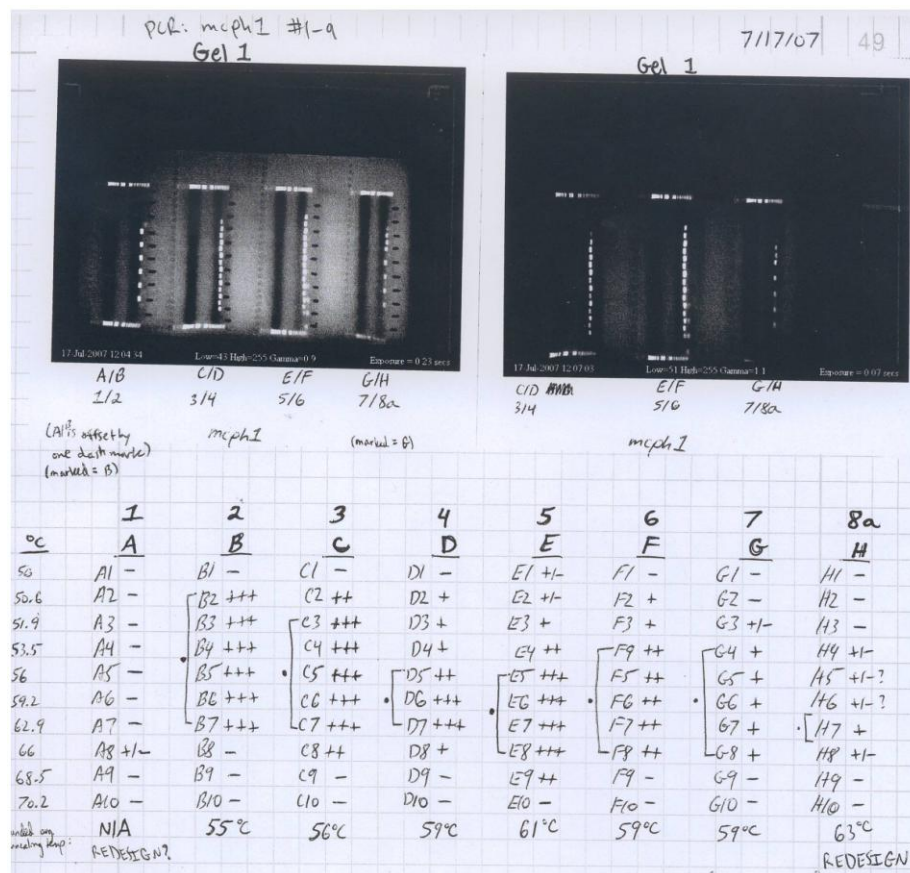


Figure 9. Example Assignment of Band Intensities. This figure shows the same gel in Figure 8 but with a list of temperatures tested and an assignment of band intensities. Each band was assigned an intensity value for that amplicon ranging from - to +++, with +++ being the strongest. The temperature gradient ran from 50-70°C.

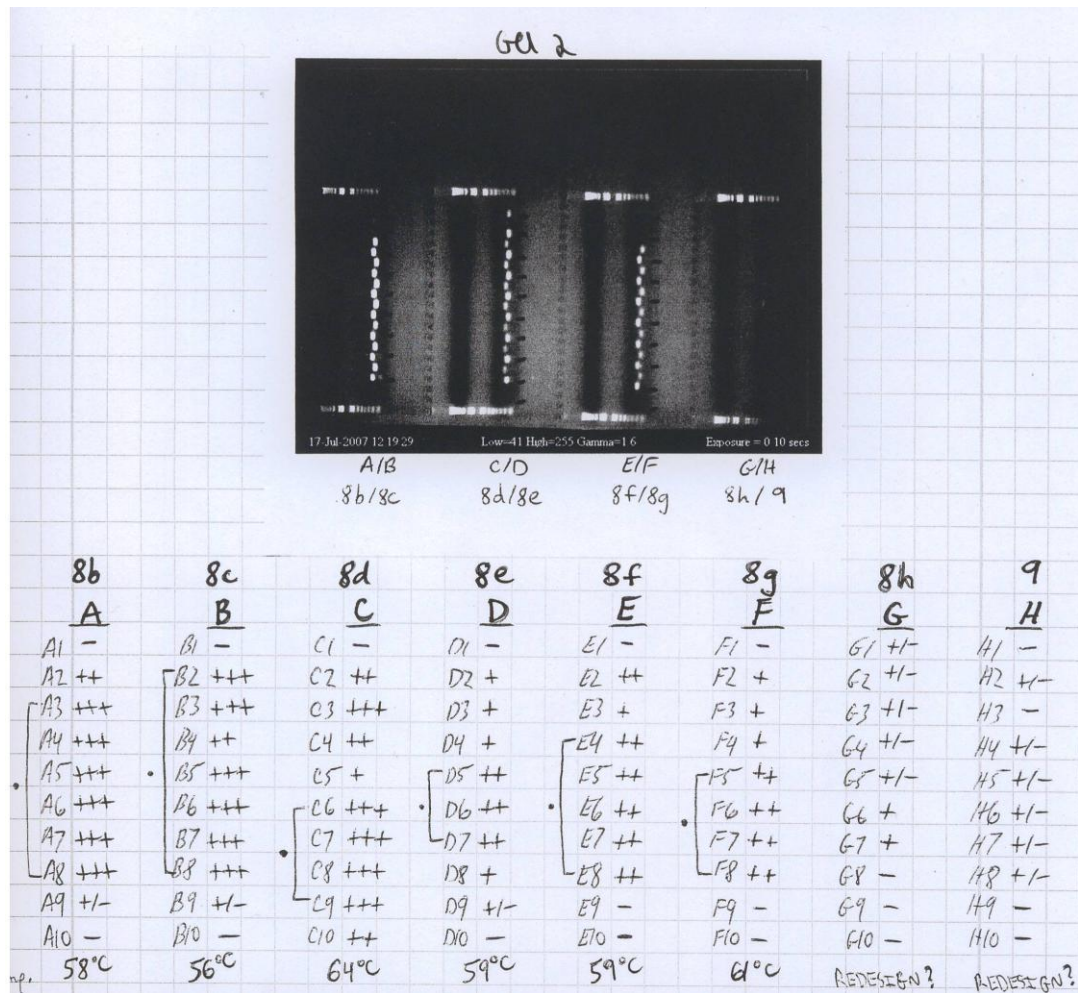


Figure 10. PCR Band Intensities for MCPH1 Exons 8b-9. Exons 8h and 9 displayed very weak bands, indicating that the primers may need to be redesigned. After running the PCR gradient on those two exons a second time, stronger bands appeared on the gel that were later optimized.

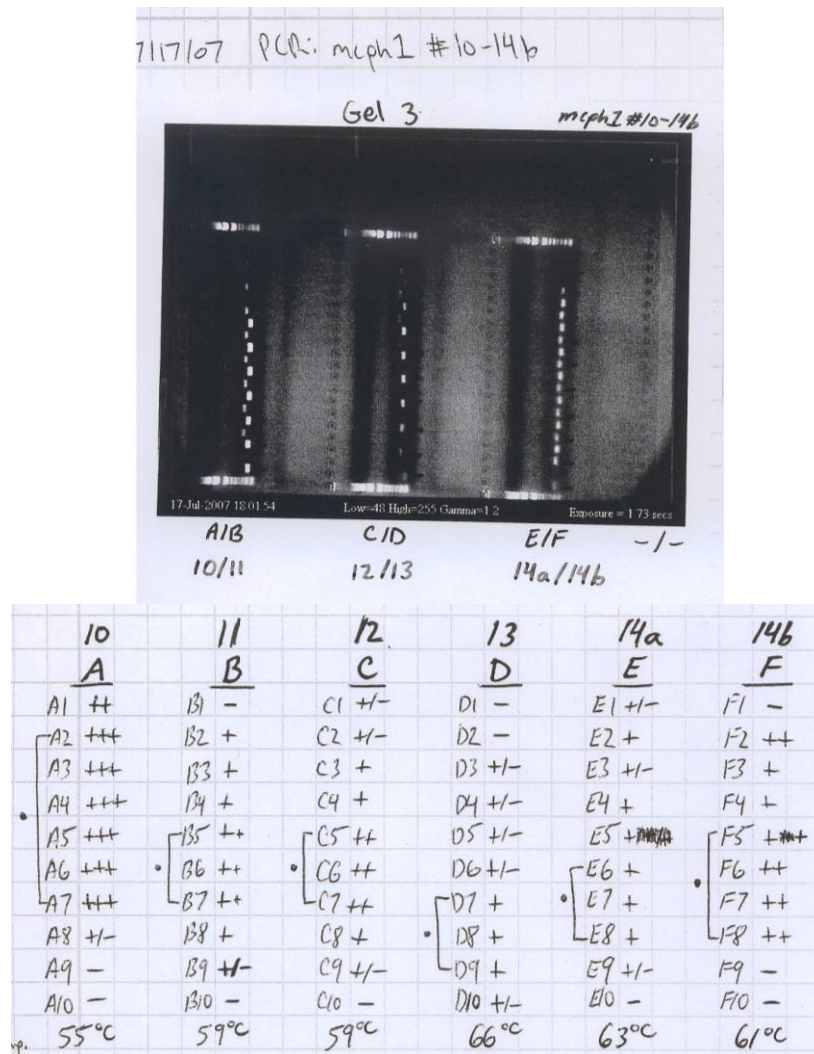


Figure 11. PCR Band Intensities for MCPH1 Exons 10-14b. The same method was used to determine the optimal primer annealing temperatures for exons 10-14b. None of these primers failed.

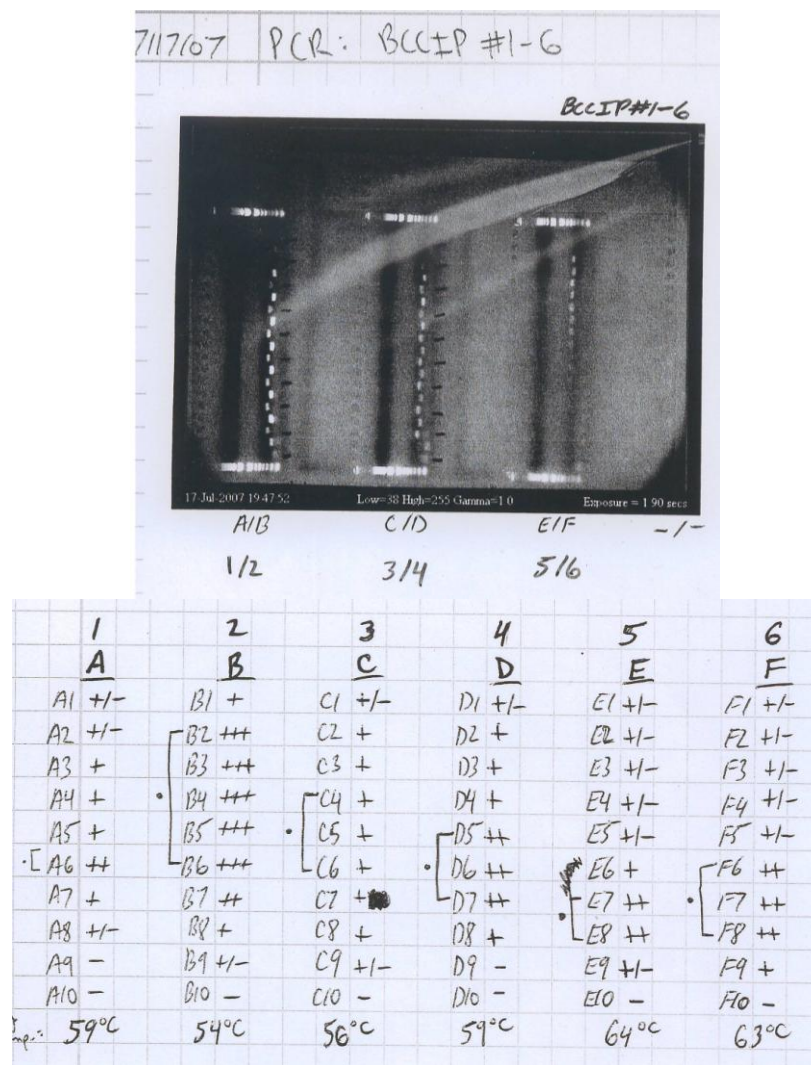


Figure 12. PCR Band Intensities for BCCIP Exons 1-6.

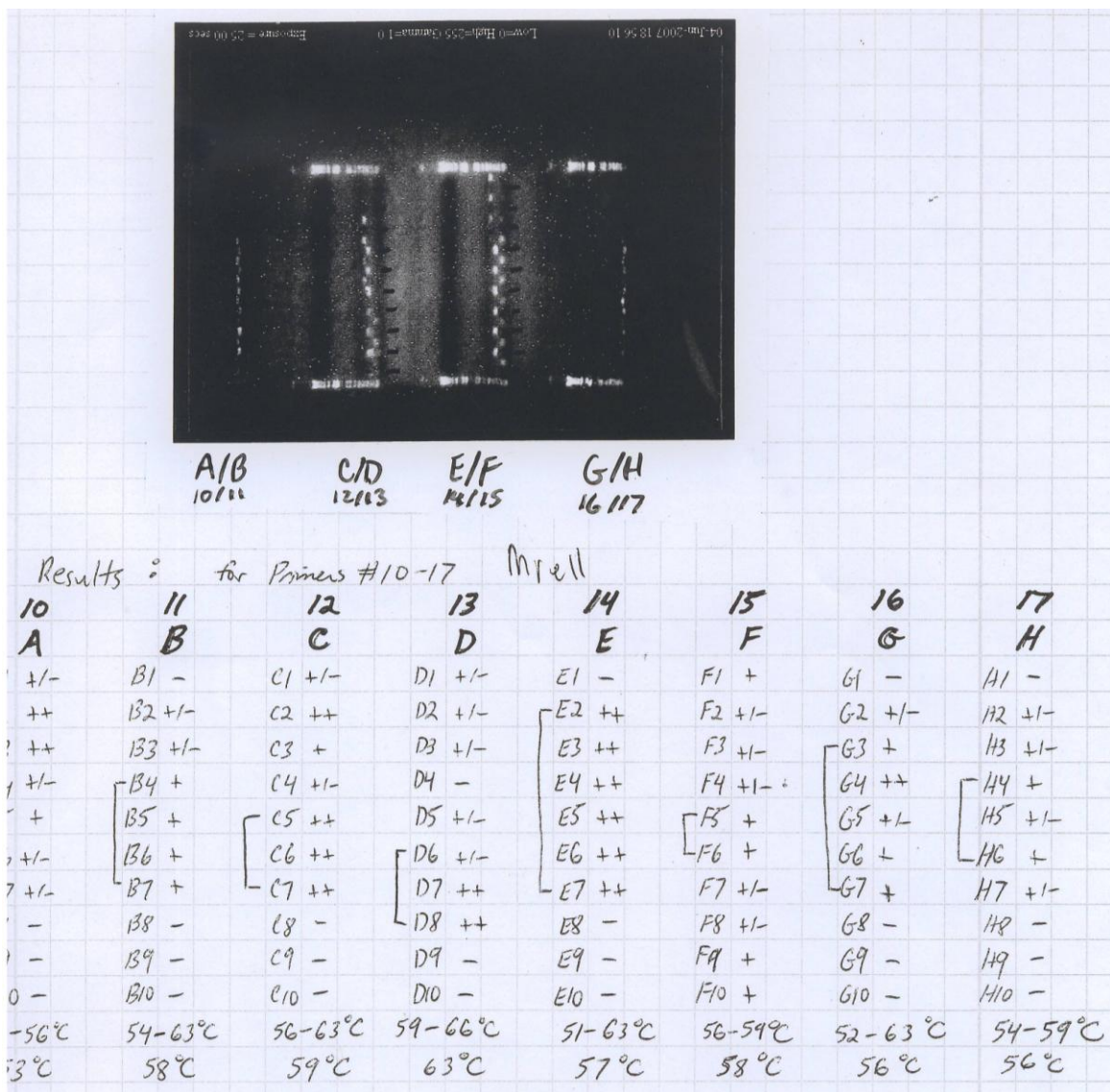


Figure 13. PCR Band Intensities for Mre11 Exons 10-17. None of the primers for these exons failed in this gel.

PCR Amplification of Candidate Genes from Patient DNA

Following primer pair optimization, the optimized PCR conditions were then applied to each of the six candidate genes from two hundred eighty-eight samples of germline DNA obtained from various collaborating centers in Europe and the U.S. Each patient had an extensive family history of breast cancer, but none had a BRCA1 or 2 mutation.

We began by individually amplifying each exon in the six target genes. Exons larger than 500 bp were split into multiple amplicons for analyses. Table-VI shows a summary of the number of amplicons successfully obtained for the six candidate genes. All 14 exons were successfully obtained for MCPH1, all 3 exons for DSS1, 19 of 20 exons for Mre11, 23 of 25 exons for Rad50, all 16-17 exons for NBS1, and all 7 exons for BCCIP were obtained.

Table-VI: Number of PCR Amplicons Obtained for Each Candidate Gene and Number of Amplicons Analyzed by HRMA.

	A	B	C	D	E	F	G	H	I
1	Gene name	#exons	#amplicons	# amplicons analyzed by HRMA	# variants identified	protein truncating	missense	silent	intronic
2	MCPH1	14	22	21					
3	DSS1	3	3	3					
4	Mre11	20	19	10					
5	Rad50	25	23	13					
6	NBS1 1	16	20	in progress					
7	NBS1 2	17	21	in progress					
8	BCCIP 1	8	11	in progress					
9	BCCIP 2	7	10	in progress					
10	BCCIP 3	7	10	in progress					
11									
12									
13									

In this Table, column-1 shows the gene analyzed; column-2 shows the total known number of exons for that gene; column-3 shows the number of amplicons analyzed for

that gene, and column-4 shows the number of amplicons analyzed by High Resolution Melting Analysis (HRMA) for that gene.

High Resolution Melting Analysis (HRMA) and Variant Sequencing

PCR amplicons for MCPH1, DSS1, Mre11, and Rad50 were analyzed by High Resolution Melting Analysis (HRMA) as a preliminary screen to identify potential sequence variants. In this technique, heteroduplexes form during PCR between one DNA strand from the WT DNA and the other strand from a patient's variant gene. Mismatched duplexes intercalate less fluorescent dye than pure duplex DNA, so a decreasing fluorescent signal indicates the presence of potential sequence variants, which can subsequently be further analyzed by DNA sequencing. HRMA Lightscanner Software was used to determine whether melting curves varied from the wild type curves to determine which of the 288 patient samples most likely contained mutations.

Table-VI (in the previous subsection) shows a summary of the number of amplicons analyzed by HRMA. All 14 exons were analyzed for MCPH1, all 3 exons for DSS1, 10 of 20 exons for MRE11, 13 of 25 exons for RAD50, and none for NBS1 or BCCIP at this time. LightScanner software was used to display the HRMA data. Any curves that visually differed from the normal (wild type) curve indicated a potential sequence variant for that patient sample. Potential variants were then analyzed by sequencing.

Of the four genes analyzed by PCR/HRMA to date, one MCPH1, was chosen for sequence analysis. A preliminary analysis of DSS1 gene is shown in the Appendix, and is nearly completed. DNAs were prepared for sequencing by using an ExoSAP IT

protocol to remove excess primers and nucleotides from the PCR reactions. The final product samples were then placed on a sequencing plate and sent to the Mayo Clinic sequencing core to be sequenced. The sequenced products were analyzed using Sequencher software, which assisted in identifying the location of a possible mutation within each amplicon.

Figure 14 shows the HRMA LightScanner output for MCPH1 Exon-6 for Patient H1. The melting curve for exon-6 of patient H1 differs from the wild-type curve, indicating a possible sequence variant. Figure 15 shows the Sequencher output for MCPH1 Exon-6 for patient E4 and H1, both showing sequence variants relative to WT DNA. Figures 16-21 show examples of other sequence variants identified.

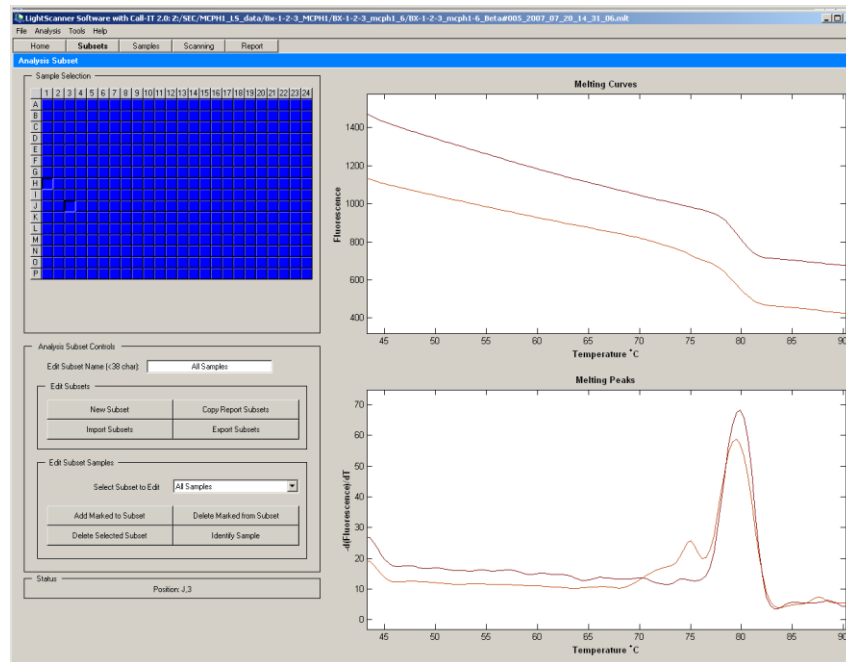


Figure 14. HRMA LightScanner Output for MCPH1 Exon-6 for Patient H1 (BR-32-515-d05-3184). The light colored double-peak curve for the patient DNA (lower right panel) differs from the darker colored single peak for WT DNA indicating a potential sequence variant.

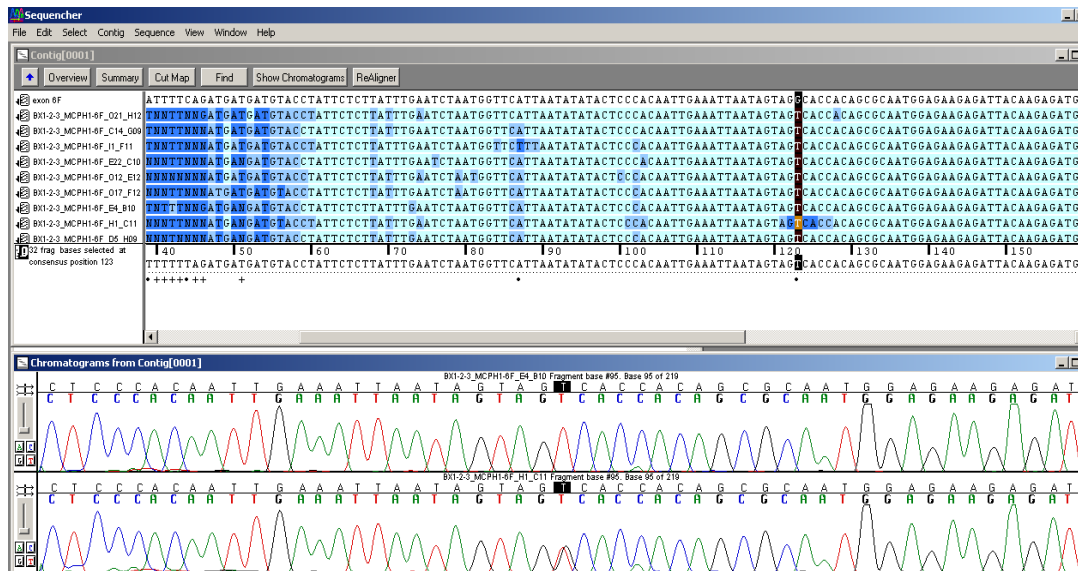


Figure 15. Sequencer Output for MCPH1 Exon-6 for Two Patients E4 (BR-32-127-d00-2063) and H1 (BR-32-515-d05-3184). The upper sequence file for patient E4 (BR-32-127-d00-2063) shows a homozygous S 171 R (TT) mutation relative to WT DNA (GG). Note: the WT sequence is not shown in this figure. The lower sequence displays a heterozygous 581 G>T mutation (GT).

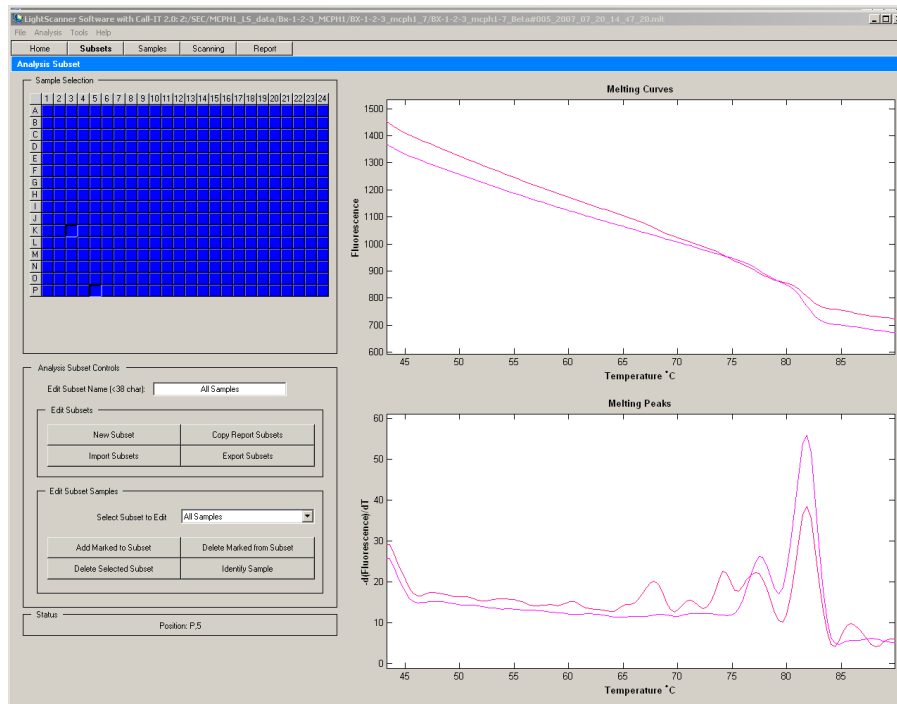


Figure 16. HRMA LightScanner Output for MCPH1 Exon-7 for Patients K3 (FCP-119) and P5 (COH-2394-469-1). This LightScanner output shows a double peak curve that is the wild type curve and a multiple peak curve that corresponds to the frameshift mutation (K3).

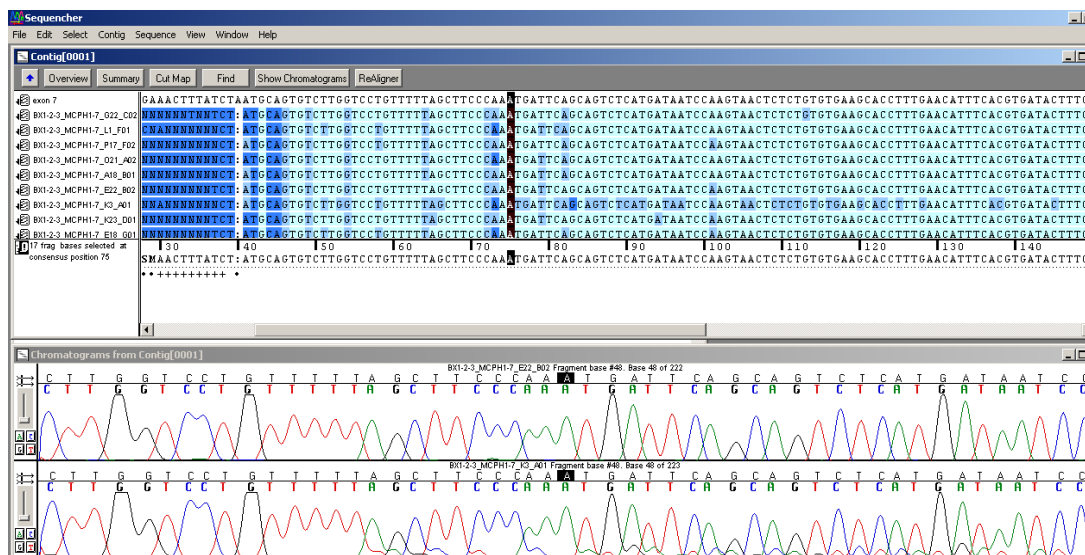


Figure 17. Sequencer Output for MCPH1 Exon-7 for Two Patients E22 (COH-1228-863-1) and K3 (FCP-119). This figure shows a frameshift mutation (Exon 7 c. 649-7 ins T, IVS 6-7 ins T) in the second sequence file relative to the WT sequence (top sequence).

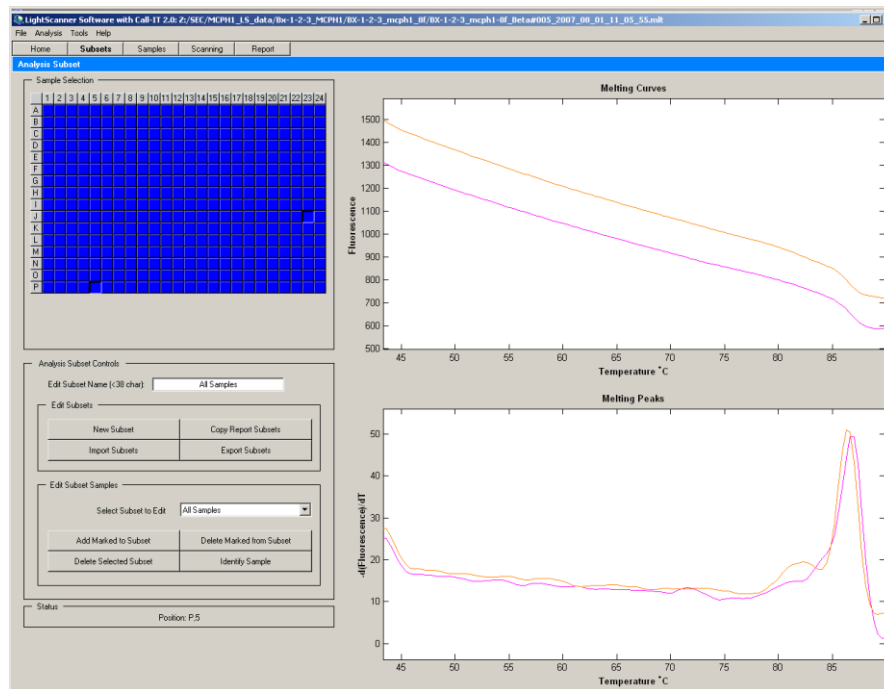


Figure 18. HRMA LightScanner Output for MCPH1 Exon-8F for Patients J23 (FCP-10) and P5 (COH-2394-469-1). This HRMA shows a pink curve wild type (P5) and an orange curve (J23) that corresponds to the frameshift mutation.

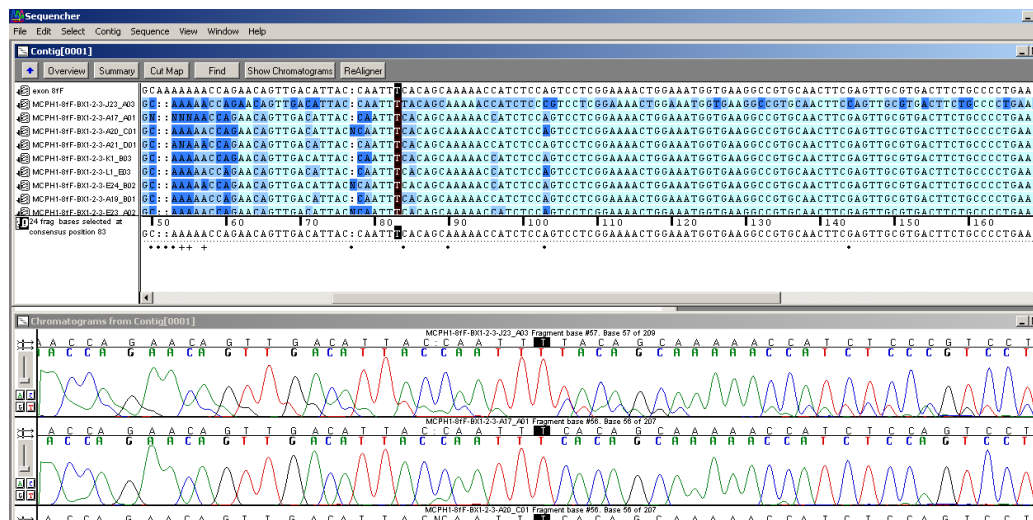


Figure 19. Sequencer Output for MCPH1 Exon-8F for Two Patients J23 (FCP-10) and A17. The first sequence file is an example of a frameshift mutation. In this sequence, there is an apparent shoulder on the peaks, which is an indication of a frameshift mutation (1464 Δ del A, fs (468) codon 466 stop 499). In this specific example, a stop codon is formed at codon 499 in exon 8f of the MCPH1 gene.

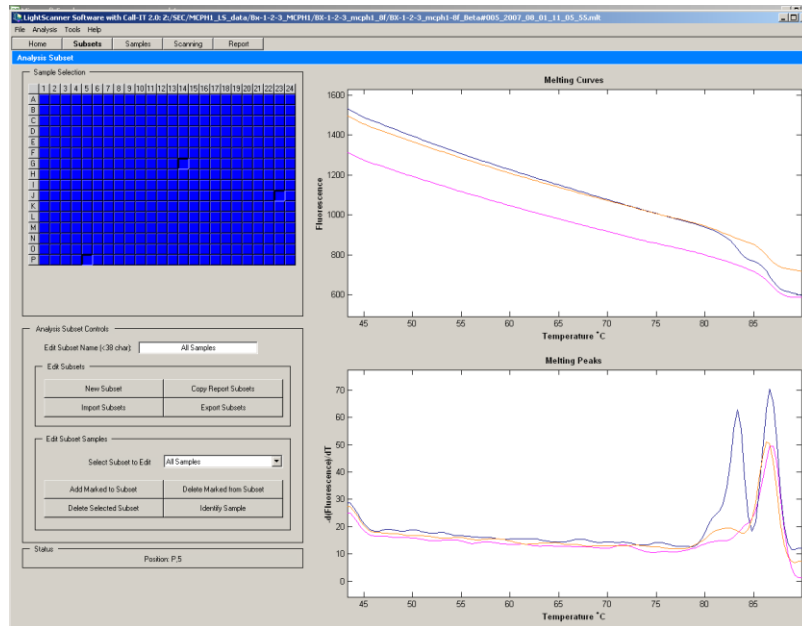


Figure 20. LightScanner Output for MCPH1 Exon-8F for Patients P5 (COH-2394-469-1), G14 (COH-883-856-1), and J23 (FCP-10). The pink curve (P5) is the wild type curve. The orange and dark blue curves (G14 and J23) are heterozygous mutations. The orange curve is also a frameshift mutation.

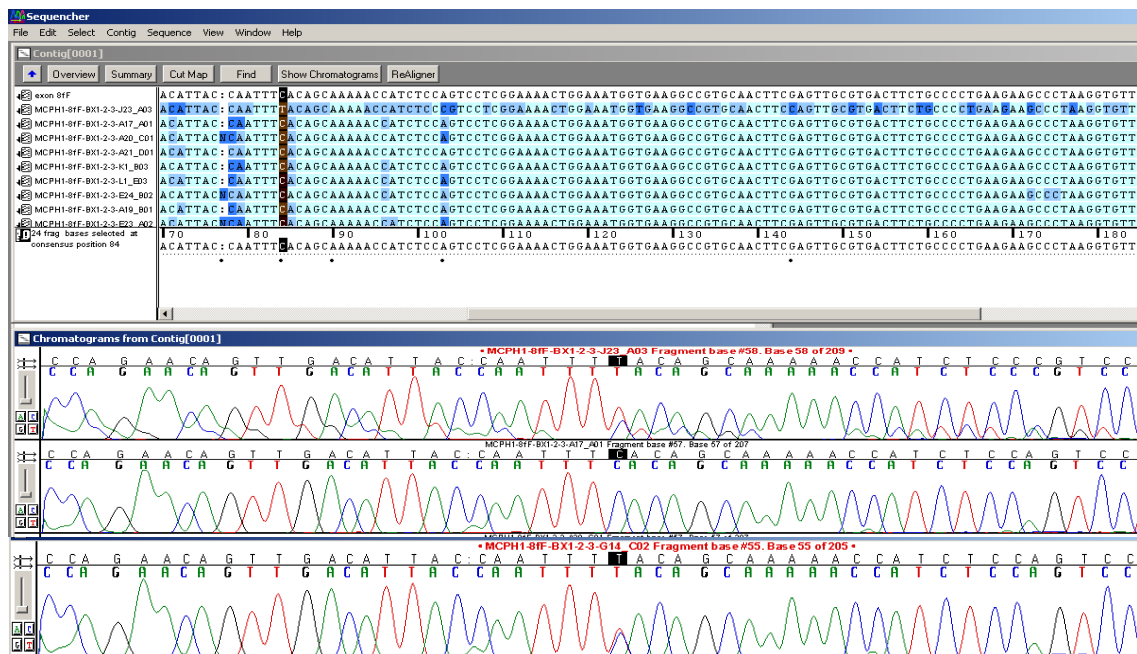


Figure 21. Sequencer Output for MCPH1 Exon-8F for Patients J23 (FCP-10), A17, and G14 (COH-883-856-1). This is another example of a frameshift mutation (1496 C>T, F 476 F). The first sequence shows a mutation of C>T at position 85 in the frameshift/mutant sequence. The mutation is homozygous. The second sequence displays the WT sequence, while the third sequence shows an individual that is heterozygous for the SNP.

In order to determine how a particular amino acid would be altered by the nucleotide mutation, the location of the nucleotide within the correct reading frame of the coding sequence was identified. Altered codons will change the resultant protein sequence, except for silent mutations where the protein is not altered or physically changed. However, silent mutations may have some function in alternative splicing.

Once the locations of the amino acid changes had been recorded, the dbSNP NCBI database was accessed to determine the frequency of the SNPs previously identified in other population studies. Since the 288 patient samples consisted of individuals from European countries, the Minor Allele Frequencies (MAFs) were calculated from the data available on European populations. Novel SNPs, previously unidentified, were not recorded in the dbSNP database. Frameshift mutations were also not available in the dbSNP database, because frameshifts alter the sequence of multiple nucleotides (rather than a single nucleotide). The sequence data analysis summary for MCPH1 is shown in Table-VII below.

Table-VII: Summary of the Sequence Analysis Mutation Screening for MCPH1.

Coordinates	exon	nt change	Genotype	aa change	dbSNP	MAF	Obs freq in X sequences (mutants/#samples sequenced)	Cross species conservation
I. Frameshifts/nonsense								
BX-1-2-3 J23	8f	1464 A del A	CT	fs (468) codon 466 stop 499	rs2920676	0.0085	1 out of 23	2/8 del, 2/8 c, 4/8 nc
BX-1-2-3 J23	8f	1464 A del A; 1496 C>T		fs (468) codon 466 stop 499; F 476 F			2 out of 23	
BX-1-2-3 K3	7	c. 649 -7 ins T		IVS 6 -7 ins T			1 out of 16	
BX-1-2-3 C5	13-1	2375 ins C		(769) fs 772 Stop 777			3 out of 48	
BX-1-2-3 E7	13-1	2375 ins C		(769) fs 772 Stop 777			3 out of 48	
BX-1-2-3 K16	13-1	2375 ins C		(769) fs 772 Stop 777			3 out of 48	
BX-1-2-3 J23	13-1	2375 A del C		(769) fs 771Stop778			1 out of 48	
BX-1-2-3 G4	13-1	del 244		fs 818 Stop 844			2 out of 48	
BX-1-2-3 D5	13-1	2283del238 (ex.13 del)		fs 818 Stop 844			2 out of 48	
II. Missense subst.								
BX-1-2-3 H1	3	237 C>A	CA	Q 57 K	No		7 out of 24	100% c
BX-1-2-3 M15	3	244 C>A	CA	T 59 N	No		1 out of 24	100% c
BX-1-2-3 O20	3	250 A>G	AG	D 61 G	No		1 out of 24	7/8 c, 1/8 nc
BX-1-2-3 I21	5	430 C>A	CA	P 121 Q	No		14 out of 24	100% c
BX-1-2-3 O21	6	581 G>T	TT	S 171 R	rs2442513	0.25	30 out of 31	3/8 c, 3/8 sc, 2/8 nc
BX-1-2-3 H1	6	581 G>T	GT	S 171 R	rs2442513	0.25	1 out of 31	3/8 c, 3/8 sc, 2/8 nc
BX-1-2-3 G14	8b	931 C>A	CA	P 288 H	rs35590577	???	1 out of 15	1/8 del, 1/8 nc, 6/8 c
BX-1-2-3 G14	8b	979 G>T	GT	R 304 I	rs2083914	0.182	10 out of 15	5/8 c, 3/8 nc
BX-1-2-3 L5	8b	1008 G>C	CC	D 314 H	rs930557	0.207	5 out of 15	1/8 del, 5/8 c, 2/8 nc
BX-1-2-3 O11	8b	1008 G>C	GC	D 314 H	rs930557	0.207	9 out of 15	1/8 del, 5/8 c, 2/8 nc
BX-1-2-3 I15	8d-1	1243 A>G	GG	D 392 G	rs2515569	1	46 out of 46	2/8 del, 1/8 c, 5/8 nc (3G, 1R, 1Q)
BX-1-2-3 M4	13-1	2350 C>T	CT	A 761 V	rs1057090	0.481	23 out of 48	5/8 c (4V,1M), 2/8 nc, 1/8 sc
BX-1-2-3 G9	13-1	2350 C>T	TT	A 761 V	rs1057090	0.481	9 out of 48	5/8 c (4V,1M), 2/8 nc, 1/8 sc
BX-1-2-3 M4	13-1	2476 C>A	CA	A 806 A	rs2912016	0.43	21 out of 48	5/8 c, 2/8 sc, 1/8 nc
BX-1-2-3 G9	13-1	2476 C>A	AA	A 806 A	rs2912016	0.43	5 out of 48	5/8 c, 2/8 sc, 1/8 nc
BX-1-2-3 H3	13-1	2287 G>A	AA	C 740 Y	No		1 out of 48	7/8 c, 1/8 nc
BX-1-2-3 B21	14	2540 C>T	TT	P 828 S	No		6 out of 47	8/8 nc (4S, 2F, 1D, 1M which is sc to S)
BX-1-2-3 M8	14	2540 C>T	TC	P 828 S	No		26 out of 47	8/8 nc (4S, 2F, 1D, 1M which is sc to S)
BX-1-2-3 D3	14	2562 C>A	CA	L 832 I	No		47 out of 47?	6/8 c (5L, 1I), 2/8 sc
III. Silent substitutions								
BX-1-2-3 A22	3	296 G>T	TT	V 76 V	rs2305022	0.202	19 out of 24	100% c
BX-1-2-3 D23	3	296 G>T	GT	V 76 V	rs2305022	0.202	4 out of 24	100% c
BX-1-2-3 I1	6	545 A>T	TA	S 159 S	rs41313948	N/A	2 out of 31	100% c
BX-1-2-3 I15	8d-1	1280 T>A	TA	A 404 A	No		out of 46	2/8 del, 4/8 c, 2/8 sc
BX-1-2-3 M4	8d-1	1280 T>A	TT?	A 404 A	No		out of 46	2/8 del, 4/8 c, 2/8 sc
BX-1-2-3 G14	8f	1496 C>T	CT	F 476 F	rs2920676	0.0085	2 out of 23	2/8 del, 2/8 c, 4/8 nc
BX-1-2-3 A21	8h	1850 G>A	GA	T 594 T	rs2584	0.36	5 out of 8	2/8 del, 3/8 c, 3/8 nc
BX-1-2-3 M20	8h	1850 G>A	GA	T 594 T	rs2584	0.36	8 out of 21	2/8 del, 3/8 c, 3/8 nc
BX-1-2-3 O20	8h	1850 G>A	AA	T 594 T	rs2584	0.36	3 out of 21	2/8 del, 3/8 c, 3/8 nc
BX-1-2-3 M4	13-1	2294 C>T	CT	S 742 S	rs2912010	0.475	22 out of 48	2/8 c, 4/8 sc, 2/8 nc
BX-1-2-3 G9	13-1	2294 C>T	TT	S 742 S	rs2912010	0.475	9 out of 48	2/8 c, 4/8 sc, 2/8 nc
BX-1-2-3 M4	13-1	2476 C>A	CA	A 806 A	rs2912016	0.43	21 out of 48	5/8 c, 2/8 sc, 1/8 nc
BX-1-2-3 G9	13-1	2476 C>A	AA	A 806 A	rs2912016	0.43	5 out of 48	5/8 c, 2/8 sc, 1/8 nc
BX-1-2-3 P7	13-1	2411 G>A	GA	L 781 L	No		1 out of 48	100% c
IV. Intronic variants								
BX-1-2-3 H3	I-9	2003+9 G>A	AG	No		1 out of 47	
BX-1-2-3 F17	I-9	2003+69 A>G	AG	No		1 out of 47	
BX-1-2-3 M20	I-10F	2041+15 T>A	TA	No		1 out of 45	
BX-1-2-3 D3	I-14	2576+8 C>A	CA	No		47 out of 47?	

Table-VII: Summary of the Sequence Analysis Mutation Screening for Gene MCPH1. The columns in this table indicate the exon number for MCPH1, the observed nucleotide number change, the genotype for the mutation, the amino acid change designation, whether the mutation has been identified in the dbSNP database, the minor allele frequency (MAF), the observation frequency of the corresponding mutant, and the cross species conservation of the mutant amino acid.

The project to date has found MCPH1 mutations in 29 of the 288 patient samples tested, 10 of which demonstrated more than one mutation. Nine likely deleterious, frameshift/nonsense mutations, nineteen missense mutations, fourteen silent substitutions, and three intronic variants were found in the MCPH1 amplicons. The mutations that are most likely disease-associated are the missense and frameshift mutations. Frameshifts, missense mutations, and silent substitutions within amplicon 13-1 (exon 13) occurred most often within each of the three categories. Six of the nine frameshifts, five of the nineteen missense mutations, and five of the fourteen silent substitutions occurred in amplicon 13-1 (exon13).

A cross species alignment was also performed for MCPH1 (Figure 22) for nine different species to determine the degree of conservation. The more highly conserved the sequence domain, the more likely that domain is required for function, thus if a mutation occurs in a conserved domain it likely is deleterious. The amino acids highlighted in red indicate the locations where mutations were observed in patient samples in the present study. The cross species conservation for each of these mutations is also listed in the rightmost column of Table-VII. We conclude from the amino acid alignments that several domains in MCPH1 are highly conserved and are likely required for function, and that 14 patient mutations were identified in these potential functional domains.

[illegible]

54

Pedigrees of Families with MCPH1 Mutations

Figures-23, 24, 25, and 26 show the family pedigrees of patient samples found to have a serious frameshift mutation in the MCPH1 gene. The males are indicated as squares, while the females are represented as circles. Patients with breast cancer are highlighted in black. The age of cancer diagnosis (in years) is indicated within the symbol and, in some cases, both ages are given for bilateral breast cancer. Other cancers noted were bladder (Bl), colon (Col), liver (Li), ovarian (Ov), pancreatic (Panc), prostate (Pr), sarcoma (Sarc), thyroid (Thy), and uterine (Ut). The lines through symbols indicate deceased family members.

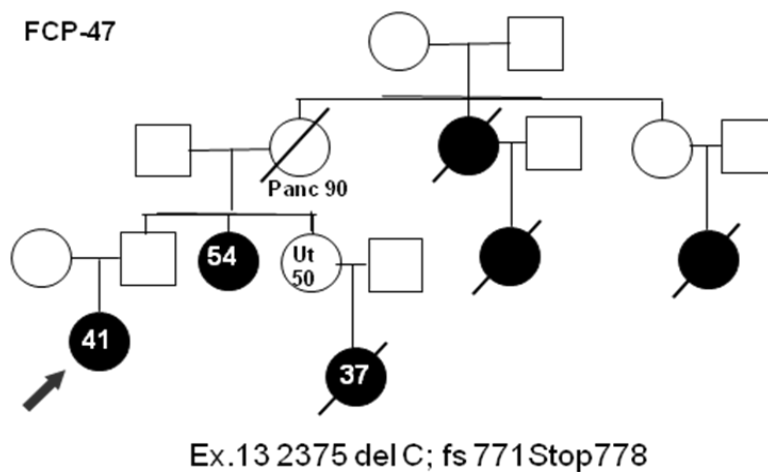


Figure 23. Pedigree of a Female Patient Identified with Breast Cancer at 41 Years of Age with an MCPH1 Frameshift Mutation.

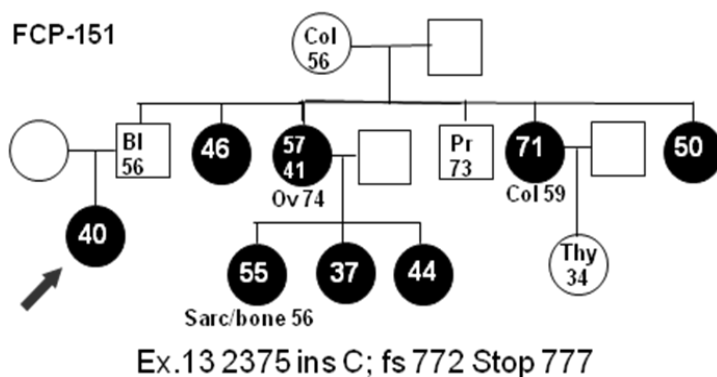


Figure 24. Pedigree of a Female Patient Identified With Breast Cancer at 40 Years of Age With an MCPH1 Frameshift Mutation.

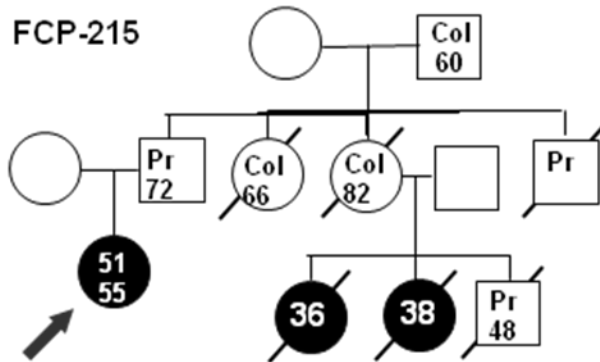


Figure 25. Pedigree of female patient identified with bilateral breast cancer first at age 51 then again at 55 years with an MCPH1 Frameshift Mutation.

Ex.13 2375 ins C; fs 772 Stop 777

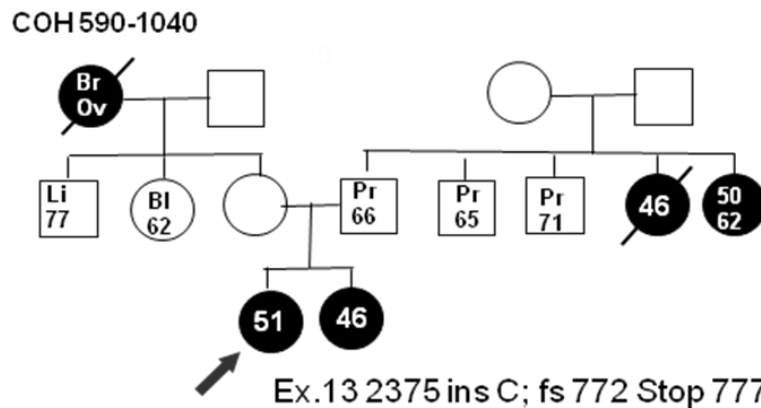


Figure 26. Pedigree of A Female Patient Identified With Breast Cancer at Age 51 with an MCPH1 Frameshift Mutation.

Ex.13 2375 ins C; fs 772 Stop 777

From these pedigrees it can be concluded that deleterious MCPH1 frameshift mutations occur in families with inherited early onset cancers. In three of the four pedigrees shown, one or more of the patient's family members died of early onset breast cancer. Further studies may eventually show how widespread these MCPH1 mutations are. Since these patients are from non-BRCA families, it can be inferred that frameshift mutations within the MCPH1 gene may have a significant effect on the onset of breast cancer.

DISCUSSION

The data obtained from this project used a candidate gene screening approach to identify potential genetic changes in proteins previously shown to interact with BRCA1 and/or BRCA2 in cellular pathways. The coding sequences of 6 candidate genes MRE11, RAD50, MCPH1, NBS1, DSS1, and BCCIP were amplified by PCR from 288 non-BRCA1/BRCA2 breast cancer patient DNA samples. The amplicons were initially screened for mutations by High Resolution Melting Analysis (HRMA), and selected positives were further analyzed by sequence analysis. Most of the data obtained so far is from gene MCPH1, which to date shows frameshift/nonsense mutations, missense mutations, silent substitutions, and intronic variants in 29 patients of the 288 patients analyzed so far. Ten patient samples demonstrated more than one mutation in MCPH1. For gene DSS1 (see Appendix), all exons for that gene were obtained by PCR, analyzed by HRMA, and sequenced, but only exon 2 has been analyzed to date. Within that amplicon, seven frameshift mutations and one SNP were found. Thus in this study to date, putative breast cancer-associated mutations have been identified in candidate genes known to interact with BRCA1 and/or BRCA2 in the double-stranded DNA break repair pathway. Importantly, the identified mutations include protein truncating and missense mutations in highly conserved domains.

The data from this project support the findings of Friedenson (2005) who previously speculated that a breakdown in the DNA repair BRCA pathway may increase the risk for development of breast cancer, and who also proposed that inactivation of any component within the BRCA pathway may also increase the risks for ovarian cancers,

lymphomas, and leukemias (Friedenson 2007). In addition, Chaplet *et al.* (2006) suggested that MCPH1 (also known as BRIT1) plays a role as a tumor suppressor. Depletion of BRIT1 (MCPH1) eliminates the DNA damage checkpoint and repair response, increasing the risk for the development and progression of cancer (Chaplet *et al.*, 2006). The majority of mutations that we found in MCPH1 were missense mutations in conserved domains, so these could disrupt function. In addition, the nine frameshift mutations observed in MCPH1 are probably serious mutations that most likely could cause disease in those patients. So perhaps a proportion of the cancer observed in non-BRCA1/BRCA2 families can be explained by these observed mutations in the MCPH1 gene.

Problems encountered while working on this project included difficulty amplifying certain exons by PCR, which led to a redesign and reoptimization of primers in many instances. In addition, the HRMA assay used as a pre-screening method to identify potential mutations was not very efficient or consistent at detecting the mutations later identified by sequencing. It was also somewhat difficult to manage the large amounts of data generated in this project, especially when analyzing so many patient samples.

In the future, database software programs may be useful for organizing the data. In addition, MCPH1 mutation segregation studies and penetrance estimation studies could be done to determine disease-risk. Additional studies could also be completed on other candidate genes to continue to identify genes that contribute to an unexplained familial risk for breast cancer.

BIBLIOGRAPHY

- Antoniou AC, Easton DF (2006) Models of Genetic Susceptibility to Breast Cancer. *Oncogene* **25**: 5898-5905.
- Breast and Ovarian Cancer (2007) *Genes and Disease*. 1 Dec 2007.
<http://www.ncbi.nlm.nih.gov/books/bv.fcgi?call=bv.View..ShowSection&rid=gnid.section.99>
- Breast Cancer Mutation Database (2007) *National Human Genome Research Institute*
 < <http://research.nhgri.nih.gov/bic/>>
- Cellular and Molecular Biology - DNA Analysis (2005) *National Center for Voice and Speech* 20 Aug 2008 www.ncvs.org/ncvs/groups/cmb/dna.html
- Chaplet M, Rai R, Jackson-Bernitsas D, Li K, Lin SY (2006) BRIT1/MCPH1: a guardian of genome and an enemy of tumors. *Cell Cycle*. 2006 Nov;5(22):2579-83. Epub 2006 Nov 15.
- Cierniková S, Tomka M, Kovac M, Stevurkova V, Bella V, JNovotny J and Zajac V (2005) Mutation screening of BRCA1, BRCA2 and CHEK2*1100delC in Slovak HBOC families. *Breast Cancer Research* **7**(Suppl 2): P1.02.
- Cipollini G, Tommasi S, Paradiso A, Aretini P, Bonatti F, Brunetti I, Bruno M, Lombardi G, Schittulli F, Sensi E, Tancredi M, Bevilacqua G, Caligo MA (2004) Genetic alterations in hereditary breast cancer. *Ann Oncol*. **15** Suppl 1: I7-I13.
- Cowden syndrome (2007) *Genetics Home Reference*. 8 Dec 2007.
<http://ghr.nlm.nih.gov/condition=cowdensyndrome>
- Csokay B, Tihomirova L, Stengrevics A, Sinicka O, Olah E (1999) Strong founder effects in BRCA1 mutation carrier breast cancer patients from Latvia. Mutation in brief no. 258. *Hum Mutat*. **14**(1): 92.
- Deng, Chu-Xia (2006) BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. *Nucleic Acids Res*. **34**(5): 1416–1426.
- De Soto JA and Deng, Chu-Xia (2006) PARP-1 inhibitors: are they the long-sought genetically specific drugs for BRCA1/2-associated breast cancers? *Int J Med Sci* **3**(4): 117–123.
- Easton DF, Ford D, Bishop DT (1995) Breast and ovarian cancer incidence in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. *Am J Hum Genet* **56**:265–71.

- Finch A, Beiner M, Lubinski J, Lynch HT, Moller P, Rosen B, Murphy J, Ghadirian P, Friedman E, Foulkes WD, Kim-Sing C, Wagner T, Tung N, Couch F, Stoppa-Lyonnet D, Ainsworth P, Daly M, Pasini B, Gershoni-Baruch R, Eng C, Olopade OI, McLennan J, Karlan B, Weitzel J, Sun P, Narod SA; Hereditary Ovarian Cancer Clinical Study Group. (2006) Salpingo-oophorectomy and the risk of ovarian, fallopian tube, and peritoneal cancers in women with a BRCA1 or BRCA2 Mutation. *JAMA*. Jul **296**(2):185-92
- Ford D, Easton DF, Stratton M, Narod S, Goldgar D, Devilee P, Bishop DT, Weber B, Lenoir G, Chang-Claude J, Sobol H, Teare MD, Struwing J, Arason A, Scherneck S, Peto J, Rebbeck TR, Tonin P, Neuhausen S, Barkardottir R, Eyfjord J, Lynch H, Ponder BA, Gayther SA, Zelada-Hedman M, et al. (1998) Genetic Heterogeneity and Penetrance Analysis of the BRCA1 and BRCA2 Genes in Breast Cancer Families. The Breast Cancer Linkage Consortium. *Am J Hum Genet*. **62**: 676-689.
- Friebel TM, Domchek SM, Neuhausen SL, Wagner T, Evans DG, Isaacs C, Garber JE, Daly MB, Eeles R, Matloff E, Tomlinson G, Lynch HT, Tung N, Blum JL, Weitzel J, Rubinstein WS, Ganz PA, Couch F, Rebbeck TR (2007) Bilateral prophylactic oophorectomy and bilateral prophylactic mastectomy in a prospective cohort of unaffected BRCA1 and BRCA2 mutation carriers. *Clin Breast Cancer*. Dec; **7**(11): 875-882.
- Friedenson B (2005) BRCA1 and BRCA2 Pathways and the Risk of Cancers Other Than Breast or Ovarian. *Med Gen Med*. **7**: 60.
- Friedenson B (2007) The BRCA1/2 pathway prevents hematologic cancers in addition to breast and ovarian cancers. *BMC Cancer* **7**: 152.
- Frank B, Hemminki K, Meindl A, Wappenschmidt B, Sutter C, Kiechle M, Bugert P, Schmutzler RK, Bartram CR, Burwinkel B (2007) BRIP1 (BACH1) variants and familial breast cancer risk: a case-control study. *BMC Cancer* **7**: 83.
- Ganguly A, Leahy K, Marshall AM, Dhulipala R, Godmilow L, Ganguly T (1997) Genetic testing for breast cancer susceptibility: frequency of BRCA1 and BRCA2 mutations. *Genet Test*. **1**(2): 85-90.
- Gorski B, Byrski T, Huzarski T, Jakubowska A, Menkiszak J, Gronwald J, Pluzańska A, Bebenek M, Fischer-Maliszewska L, Grzybowska E, Narod SA, Lubiński (2000) Founder mutations in the BRCA1 gene in Polish families with breast-ovarian cancer. *Am J Hum Genet*. Jun; **66**(6):1963-8. Epub 2000 Apr 28.
- Górski B, Jakubowska A, Huzarski T, Byrski T, Gronwald J, Grzybowska E, Mackiewicz A, Stawicka M, Bebenek M, Sorokin D, Fiszer-Maliszewska Ł, Haus O, Janiszewska H, Niepsuj S, Gózdź S, Zaremba L, Posmyk M, Pluzańska M, Kilar

- E, Czudowska D, Waśko B, Miturski R, Kowalczyk JR, Urbański K, Szwiec M, Koc J, Debniak B, Rozmiarek A, Debniak T, Cybulski C, Kowalska E, Tołoczko-Grabarek A, Zajaczek S, Menkiszak J, Medrek K, Masojć B, Mierzejewski M, Narod SA, Lubiński J. (2004) A high proportion of founder BRCA1 mutations in Polish breast cancer families. *Int J Cancer*. **110**(5): 683-686.
- Greene MH (1997) Genetics of breast cancer. *Mayo Clin Proc*. **72**(1): 54-65.
- Gronwald J, Elsakov P, Górski B, Lubiński J (2005) High incidence of 4153delA BRCA1 gene mutations in Lithuanian breast- and breast-ovarian cancer families. *Breast Cancer Res Treat*. **94**(2): 111-113.
- Hartmann LC, Sellers TA, Schaid DJ, Frank TS, Soderberg CL, Sitta DL, Frost MH, Grant CS, Donohue JH, Woods JE, McDonnell SK, Vockley CW, Deffenbaugh A, Couch FJ, Jenkins RB (2001) Efficacy of bilateral prophylactic mastectomy in BRCA1 and BRCA2 gene mutation carriers. *J Natl Cancer Inst*. **93**(21): 1633-1637.
- Hall JM, Lee MK, Newman B, Morrow JE, Anderson LA, Huey B, King MC (1990) Linkage of early-onset familial breast cancer to chromosome 17q21. *Science*. **250**(4988): 1684-1689.
- Heikkinen K, Rapakko K, Karppinen SM, Erkkö H, Knuutila S, Lundán T, Mannermaa A, Børresen-Dale AL, Borg A, Barkardóttir RB, Petrini J, Winqvist R (2006) RAD50 and NBS1 are breast cancer susceptibility genes associated with genomic instability. *Carcinogenesis*. **27**(8): 1593-1599.
- Hermon C, Beral V (1996) Breast cancer mortality rates are levelling off or beginning to decline in many western countries: analysis of time trends, age-cohort and age-period models of breast cancer mortality in 20 countries. *Br J Cancer*. **73**(7): 955-960.
- Kainu T, Juo SHH, Desper R, Schäffer A, Gillanders E, Rozenblum E, Freas-Lutz D, Weaver D, Stephan D, Bailey-Wilson J, Kallioniemi O, Tirkkonen M, Syrjäkoski K, Kuukasjärvi T, Koivisto P, Karhu R, Holli K, Arason A, Johannesdóttir G, Bergthorsson J, Johannsdóttir H, Egilsson V, Barkardóttir R, Johannsson O, Haraldsson K, Sandberg T, Holmberg E, Grönberg H, Olsson H, Borg A, Vehmanen P, Eerola H, PHeikkilä P, Pyrhönen S, and Nevanlinna H (2000) Somatic deletions in hereditary breast cancers implicate 13q21 as a putative novel breast cancer susceptibility locus. *Proc Natl Acad Sci USA* **97**: 9603-9608.
- Knudson AG Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA* **68**: 820-823.
- Lewis AG, Flanagan J, Marsh A, Pupo GM, Mann G, Spurdle AB, Lindeman GJ, Visvader JE, Brown MA, Chenevix-Trench G; Kathleen Cuninghame Foundation

- Consortium for Research into Familial Breast Cancer (2005) Mutation analysis of FANCD2, BRIP1/BACH1, LMO4 and SFN in familial breast cancer. *Breast Cancer Res.* **7**(6): R1005-1016.
- Li J, Zou C, Bai Y, Wazer DE, Band V, Gao Q (2006) DSS1 is required for the stability of BRCA2. *Oncogene* **25**(8): 1186-1194.
- Li-Fraumeni syndrome (2007) Genetics Home Reference. 8 Dec 2007.
<http://ghr.nlm.nih.gov/condition=lifraumenisyndrome>
- Lin SY, Rai R, Li K, Xu ZX, Elledge SJ (2005) BRIT1/MCPH1 is a DNA damage responsive protein that regulates the Brca1-Chk1 pathway, implicating checkpoint dysfunction in microcephaly. *Proc Natl Acad Sci USA*. **18**;102(42): 15105-15109.
- Liu Y, West SC (2002) Distinct Functions of BRCA1 and BRCA2 in Double-Strand Break Repair. *Breast Cancer Res.* **4**: 9-13.
- Lu H, Yue J, Meng X, Nickoloff JA, Shen Z (2007) BCCIP regulates homologous recombination by distinct domains and suppresses spontaneous DNA damage. *Nucleic Acids Res.* [Epub ahead of print]
- Miki Y, Swensen J, Schattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu QY, et al (1994) Isolation of BRCA1, the 17q-linked breast and ovarian cancer susceptibility gene. *Science* **266**: 66–71.
- National Cancer Institute (2007) Breast Cancer. 4 Dec 2007.
<http://www.cancer.gov/cancertopics/types/breast>
- Nusbaum R, Isaacs C (2007) Management updates for women with a BRCA1 or BRCA2 mutation. *Mol Diagn Ther.* **11**(3): 133-144.
- Oncogenes and Tumor Suppressor Genes (2005) *American Cancer Society*. 24 Feb 2005
http://www.cancer.org/docroot/ETO/content/ETO_1_4x_oncogenes_and_tumor_suppressor_genes.asp 20 Aug 2008.
- Osborne C, Wilson P, Tripathy D (2004) Oncogenes and Tumor Suppressor Genes in Breast Cancer: Potential Diagnostic and Therapeutic Applications. *The Oncologist* **9** (4) 361–377.
- Peto J, Collins N, Barfoot R, Seal S, Warren W, Rahman N, et al. (1999) Prevalence of BRCA1 and BRCA2 gene mutations in patients with early-onset breast cancer. *J Natl Cancer Inst* **91**: 943-949.
- Peutz–Jeghers syndrome (2007) Genetics Home Reference. 8 Dec 2007.
<http://ghr.nlm.nih.gov/condition=peutzjegherssyndrome>

PC Green Melting Dyes (2007) *Idaho Technology Inc.* 21 Aug 2008
<http://www.idahotech.com/LCGreen/index.html>

Pohlreich P, Zikan M, Stribrna J, Kleibl Z, Janatova M, Kotlas J, Zidovska J, Novotny J, Petruzalka L, Szabo C, Matous B (2005) High proportion of recurrent germline mutations in the BRCA1 gene in breast and ovarian cancer patients from the Prague area. *Breast Cancer Res.* **7**(5): R728-736. Epub 2005 Jul 19.

Rai R, Dai H, Multani AS, Li K, Chin K, Gray J, Lahad JP, Liang J, Mills GB, Meric-Bernstam F, Lin SY (2006) BRIT1 regulates early DNA damage response, chromosomal integrity, and cancer. *Cancer Cell.* **10**(2): 145-157.

Robert F, Hardy S, Nagy Z, Baldeyron C, Murr R, Dery U, Masson JY, Papadopoulou D, Herceg Z, Tora L (2006) The Transcriptional Histone Acetyltransferase Cofactor TRRAP Associates with the MRN Repair Complex and Plays a Role in DNA Double-Strand Break Repair. *Mol Cell Biol.* Jan;**26**(2): 402-412.

Saxena S, Chakraborty A, Kaushal M, Kotwal S, Bhatnager D, Mohil RS, Chintamani C, Aggarwal AK, Sharma VK, Sharma PC, Lenoir G, Goldgar DE, Szabo CI (2006) Contribution of germline BRCA1 and BRCA2 sequence alterations to breast cancer in Northern India. *BMC Med Genet.* **7**: 75.

Schubert E, Lee M, Mefford H, Argonza R, Morrow J, Hull J, Dann J, King M (1997) BRCA2 in American Families with Four or More Cases of Breast or Ovarian Cancer: Recurrent and Novel Mutations, Variable Expression, Penetrance, and the Possibility of Families Whose Cancer Is Not Attributable to BRCA1 or BRCA2. *Am J Hum Genet.* **60**: 1031-1040.

Seal S, Thompson D, Renwick A, Elliott A, Kelly P, Barfoot R, Chagtai T, Jayatilake H, Ahmed M, Spanova K, North B, McGuffog L, Evans DG, Eccles D; Breast Cancer Susceptibility Collaboration (UK), Easton DF, Stratton MR, Rahman N (2006) Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles. *Nat Genet.* **38**(11): 1239-1241.

Southey MC, Tesoriero AA, Andersen CR, Jennings KM, Brown SM, Dite GS, Jenkins MA, Osborne RH, Maskiell JA, Porter L, Giles GG, McCredie MR, Hopper JL, Venter DJ (1999) BRCA1 mutations and other sequence variants in a population-based sample of Australian women with breast cancer. *Br J Cancer* **79**(1): 34-39.

Struewing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M, Timmerman M, Brody L, Tucker M (1997) The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. *N Engl J Med* **336**: 1401-1408.

- Szabo CI, King MC (1997) Population genetics of BRCA1 and BRCA2. *Am J Hum Genet.* **60**(5): 1013-1020.
- Szabo CI, Wagner LA, Francisco LV, Roach JC, Argonza R, King MC, Ostrander EA (1996) Human, canine and murine BRCA1 genes: sequence comparison among species. *Hum Mol Genet.* **5**(9): 1289-1298.
- Thompson D, Szabo CI, Mangion J, Oldenburg RA, Odefrey F, Seal S, Barfoot R, Kroeze-Jansema K, Teare D, Rahman N, Renard H, Mann G, Hopper JL, Buys SS, Andrulis IL, Senie R, Daly MB, West D, Ostrander EA, Offit K, Peretz T, Osorio A, Benitez J, Nathanson KL, Sinilnikova OM, Oláh E, Bignon YJ, Ruiz P, Badzioch MD, Vasen HF, Futreal AP, Phelan CM, Narod SA, Lynch HT, Ponder BA, Eeles RA, Meijers-Heijboer H, Stoppa-Lyonnet D, Couch FJ, Eccles DM, Evans DG, Chang-Claude J, Lenoir G, Weber BL, Devilee P, Easton DF, Goldgar DE, Stratton MR; KConFab Consortium.(2002) Evaluation of linkage of breast cancer to the putative BRCA3 locus on chromosome 13q21 in 128 multiple case families from the Breast Cancer Linkage Consortium. *Proc Natl Acad Sci U S A.* Jan 22; **99**(2): 827-831. Epub 2002 Jan 15.
- Tischkowitz M, Xia B, Sabbaghian N, Reis-Filho JS, Hamel N, Li G, van Beers EH, Li L, Khalil T, Quenneville LA, Omeroglu A, Poll A, Lepage P, Wong N, Nederlof PM, Ashworth A, Tonin PN, Narod SA, Livingston DM, Foulkes WD (2007) Analysis of PALB2/FANCN-associated breast cancer families. *Proc Natl Acad Sci USA.* **104**(16): 6788–6793.
- Tauchi H, Matsuura S, Kobayashi J, Sakamoto S, Komatsu K (2002) Nijmegen breakage syndrome gene, NBS1, and molecular links to factors for genome stability. *Oncogene.* Dec 16;**21**(58): 8967-8980.
- Troudi W, Uhrhammer N, Ben Romdhane K, Sibille C, Mahfoudh W, Chouchane L, Ben Ayed F, Bignon YJ, Ben Ammar Elgaai A (2007) Immunolocalization of BRCA1 protein in tumor breast tissue: prescreening of BRCA1 mutation in Tunisian patients with hereditary breast cancer? *Eur J Histochem.* **51**: 219-226.
- Uyei A, Peterson SK, Erlichman J, Broglio K, Yekell S, Schmeler K, Lu K, Meric-Bernstam F, Amos C, Strong L, Arun B (2006) Association between clinical characteristics and risk-reduction interventions in women who underwent BRCA1 and BRCA2 testing: a single-institution study. *Cancer* **107**: 2745-2751.
- Vogel K, Atchley D, Erlichman J, Broglio K, Ready K, V. Valero, Amo C, Hortobagyi G, Lu K, and Arun B (2007) BRCA1 and BRCA2 Genetic Testing in Hispanic Patients: Mutation Prevalence and Evaluation of the BRCAPRO Risk Assessment Model *J. Clin. Oncol.* **25**(29): 4635-4641.
- Walsh T, Casadei S, Coats KH, Swisher E, Stray SM, Higgins J, Roach KC, Mandell J, Lee MK, Ciernikova S, Foretova L, Soucek P, King MC (2006) Spectrum of

- mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer. *JAMA* **22**: 1379-1388.
- Walsh T, King M (2007) Ten Genes for Inherited Breast Cancer. *Cancer Cell*. **11**: 103-105.
- Warner E, Foulkes W, Goodwin P, Meschino W, Blondal J, Paterson C, Ozcelik H, Goss P, Allingham-Hawkins D, Hamel N, Di Prospero L, Contiga V, Serruya C, Klein M, Moslehi R, Honeyford J, Liede A, Glendon G, Brunet JS, Narod S (1999) Prevalence and penetrance of BRCA1 and BRCA2 gene mutations in unselected Ashkenazi Jewish women with breast cancer. *J Natl Cancer Inst*. **21**: 1241-1247.
- Weischer M, Bojesen SE, Tybjaerg-Hansen A, Axelsson CK, Nordestgaard BG (2007) Increased risk of breast cancer associated with CHEK2*1100delC. *J Clin Oncol*. **25**(1): 57-63. Epub 2006 Jul 31.
- Wooster R, Neuhausen S, Manigion J, Quirk Y, Ford D, Collins N, Nguyen K, et al (1994) Localisation of a breast cancer susceptibility gene (BRCA2) to chromosome 13q by genetic linkage analysis. *Science* **265**: 2088–2090.

Appendix

Sequence Analysis of DSS1

For gene DSS1, all exons for that gene were obtained by PCR, analyzed by HRMA, and sequenced, but only the data for exon-2 has been analyzed to date. Within exon-2 (Table-VIII, upper half), seven frameshift mutations and one SNP were found in 8 patient samples.

Table-VIII. Sequence Analysis of DSS1.

DSS1 Mutation Screening on BX-1-2-3									
coord	Seq folder	sequencer project	exon	seq file nt	nt change	genotype	aa change	dbSNP?	Obs freq in X sequences (mutants/# samples sequenced)
BX-1-2-3 M20	111307_be_2	2	2	fs until nt 90	FRAMESHIFT		FRAMESHIFT		2 out of 11
BX-1-2-3 I4	111307_be_2	2	2	fs until nt 90	FRAMESHIFT		FRAMESHIFT		2 out of 11
BX-1-2-3 C2	111307_be_2	2	2	fs until nt 156	FRAMESHIFT		FRAMESHIFT		1 out of 11
BX-1-2-3 G4	111307_be_2	2	2	fs until nt 160	FRAMESHIFT		FRAMESHIFT		1 out of 11
BX-1-2-3 K16	111307_be_2	2	2	fs until nt 158	FRAMESHIFT		FRAMESHIFT		1 out of 11
BX-1-2-3 J5	111307_be_2	2	2	fs until nt 114	FRAMESHIFT		FRAMESHIFT		1 out of 11
BX-1-2-3 L5	111307_be_2	2	2	fs until nt 104	FRAMESHIFT		FRAMESHIFT		1 out of 11
BX-1-2-3 I4	111307_be_2	2	2	232	284 C>T	CT	F 52 F	no	1 out of 11

This table contains the sequence analysis performed to date for DSS1. The data for exon-2 are shown in this table. Identification of the variants is currently in progress. Frameshift mutations have been positively identified within exon-2 for seven patient samples, and one patient sample displayed a SNP within the same amplicon.

The DSS1 data obtained to date support Li *et al.*'s (2006) suggestion that DSS1 depletion causes hypersensitivity to DNA damage, similar to that shown with BRCA2 mutations. These authors found DSS1 to be essential to the stability of the BRCA2 protein in mammalian cells, and suggested that its deletion, suppression, or mutation,

would promote human breast and ovarian cancer, as well as sporadic and familial breast cancer where BRCA1 and BRCA2 mutations are absent (Li *et al.* 2006).